ABSTRACT: Streptomycin and ethylene diamine tetraacetic acid (EDTA) were used to examine the role of extracellular calcium in stretch-induced muscle injury. Streptomycin was injected in one group of rats, three times daily for 8 days (S, 300 $mg.kg^{-1}.day^{-1}$ intraperitoneally). In another group, EDTA was administered (150 $mg.kg^{-1}$ IP) 20 min before and 24 h after the injury protocol. Untreated rats (C) served as controls. Muscle injury was produced by 40 stretches of active dorsiflexor muscles by ankle rotation from 80° to 130° (velocity 1.75 $rad.s^{-1}$). Ten minutes after the injury protocols, all animals lost the same amount of isometric force at both low and high stimulation frequencies (20 Hz; S, 56 ± 6%; EDTA, 47 ± 7%; C, 55 ± 4%) and 120 Hz; S, 11 ± 3%, EDTA, 13 ± 3%; C, 11 ± 3%). Tibialis anterior (TA) muscles were removed after 48 h for morphometric analysis. In both streptomycin-and EDTA-treated rats, the percent of injured (i.e., desmin-negative) myofibers in TA was reduced compared to untreated, injured muscles (S, 0.35 ± 0.08%; EDTA, 0.64 ± 0.19%; C, 1.81 ± 0.43%). Thus, streptomycin and EDTA treatment did not alter the development of muscle weakness (i.e., isometric force deficit), but almost abolished the histopathologic changes. This study shows that the mechanisms for muscle weakness and histopathologic changes (inflammation) following repeated muscle strains can largely be dissociated from each other and helps explain why there is no correlation between isometric force deficits and the number of pathologic cells.
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STREPTOMYCIN AND EDTA DECREASE THE NUMBER OF DESMIN-NEGATIVE FIBERS FOLLOWING STRETCH INJURY

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Calcium-mediated cell injury and cell death are common responses to cell membrane damage, often leading to necrosis.[31] Forty-eight hours after stretch-induced injury, necrotic myofibers can be seen in skeletal muscles.[19] Elevated intracellular calcium levels could lead to myofiber necrosis[16] by stimulating degradative pathways, including the activation of calpains[6] and phospholipases.[10] Elevated intracellular calcium levels have been reported for skeletal muscles that have been injured by lengthening contractions.[21,25,32,34]

Several studies have succeeded in attenuating muscle injury from lengthening contractions by manipulating calcium. For example, the amount of myofiber damage was markedly reduced in rats after downhill walking by administration of the nonspecific calcium chelator, ethylene diamine tetraacetic acid (EDTA), or a calcium-channel blocker, verapamil.[9] Since mitochondrial calcium concentration can be used as an indicator of elevated myofiber calcium content,[28] reduced mitochondrial calcium concentrations 1–2 days after injury in rats treated with either EDTA or verapamil provided evidence that intracellular calcium was reduced. In humans following repeated eccentric muscle actions, muscle damage was also reduced when the calcium-channel blocker, amlodipine, was administered.[5]

The exact mechanism for the elevation of myofiber calcium after lengthening contractions remains unknown. Several investigators have suggested that influx of calcium might occur through stretch-acti-

Abbreviations: ANOVA, analysis of variance; E–C, excitation–contraction; EDTA, ethylene diamine tetraacetic acid; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; NCX3, Na/Ca exchanger; TA, tibialis anterior
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vated ion channels,[6,22,38] although evidence for direct membrane damage has also been reported.[23] Using streptomycin, a known blocker of stretch-activated ion channels in cardiac muscle,[14] membrane depolarization resulting from stretch-induced injury was dramatically reduced,[22] providing evidence that direct membrane damage was not the sole source of calcium influx. Thus, there are experimental data linking stretch-induced calcium channels and muscle damage,[1] which may even be exacerbated in disease states such as muscular dystrophy.[37]

The purpose of this study was to test whether streptomycin attenuates the amount of muscle injury to the same degree as EDTA in female rat muscles exposed to repeated stretches (lengthening contractions) of activated skeletal muscles. Muscle injury was documented by a loss in maximal isometric force remaining 10 minutes after the protocol and by histopathologic changes revealed by the presence of desmin-negative myofibers 48 hours later. The results provide evidence that blockage of stretch-induced calcium entry by streptomycin almost completely abolished the histopathologic changes to the same extent as EDTA treatment but did not reduce the loss of strength following a series of repeated muscle strains.

METHODS

Animal Care and Preparation. All experimental procedures and housing conditions were approved by the West Virginia University Animal Care and Use Committee. The use of female Sprague–Dawley rats complied with Animal Welfare Act PL 99-158 and the guidelines of the Department of Health and Human Services governing the care and use of laboratory animals. Experiments were performed on three groups of rats: (1) streptomycin treated; (2) EDTA-treated; and (3) untreated control rats. Streptomycin sulfate (S-6501, Sigma Chemical Co., St. Louis, MO) was injected three times daily for 8 days [300 $mg.kg^{-1}.day^{-1}$ intraperitoneally (IP)] prior to the injury protocol [S; $n = 6$; body weight, 241 ± 3 g; age, 108 ± 11 days (mean ± SE)]. This dose was based on preliminary data. EDTA (E-478, Fisher Scientific Co., Fair Lawn, NJ) was administered (150 $mg.kg^{-1}$ IP, $n = 6$) 20 min before and 24 h after the injury protocol[9] (EDTA; $n = 6$; body weight, 264 ± 7 g; age, 140 ± 12 days). Untreated rats served as controls (C; $n = 6$; body weight, 257 ± 5 g; age, 142 ± 14 days) and were not injected.

Dynamometer testing (details described in what follows) occurred on day 7 in the streptomycin treatment group and 20 minutes after the first injection in the EDTA treatment group. Control rats did not receive injections. All rats were exposed to the injury protocol.

Before nerve cuff insertion and during all muscle testing, rats were anesthetized with isoflurane by inhalation (SurgiVet, Inc., Waukesha, WI). The level of anesthesia was administered to ensure that the hindlimb withdrawal reflex was suppressed with squeezing of the foot. Details of the dissection procedure for nerve-cuff placement, necessary for electrical stimulation of the tibial nerve of the left hindlimb, positioning of the rats, and use of the dynamometer, have been described elsewhere.[35] Following nerve-cuff placement, nerves innervating the plantar flexor (antagonist) muscles were cut. Muscle force was recorded as a reaction force under the sole of the foot in response to nerve stimulation.

Brief isometric contractions were used to determine the voltage for maximal force production. Voltage [4.3 ± 0.3 V (mean ± SE)] and pulse duration (0.2 ms) were then kept constant for each muscle preparation. After functional testing (see later), rats were placed back in their cages with water and food ad libitum until they were killed for tissue sampling.

Force–Frequency Measurements. In all groups, isometric force was measured before and following a 10-min rest after the injury protocol of lengthening contractions. Force measurements were performed at an ankle position of 130° (i.e., long muscle length) with a stimulation frequency of 20 Hz and 120 Hz. Different train durations and sufficient rest times (1500 ms, 1 min at 20 Hz and 600 ms, 2 min at 120 Hz) were used to guarantee the recording of maximal force at each stimulation frequency and to minimize fatigue. Isometric force deficits were taken as indirect evidence of functional muscle injury by lengthening contractions.[4]

Injury Protocol. A single bout of 40 lengthening contractions (stretches) of the left dorsiflexor muscles was performed by rotating the ankle at an angular velocity of 1.75 $rad.s^{-1}$ ($100°.s^{-1}$) from an ankle position of 1.40 rad (80°; i.e., short muscle length) to 2.27 rad (130°; i.e., long muscle length) with inter-contraction rest times of 40 s. Ankle position was defined as the angle between the tibia and the plantar surface of the foot, with 3.14 rad (180°) representing a completely extended foot. Each lengthening contraction was imposed after a build-up of isometric force for about 300 ms and lasted 500 ms using a stimulation frequency of 120 Hz.

Muscle Sampling. Tibialis anterior muscles from the left (injured) and right (uninjured) hindlimbs

were excised 2 days after the injury protocol to maximize the probability of observing histopathologic changes.[19] Muscles were trimmed of fat and weighed. The distal part of the tibialis anterior (TA) muscles (5–10 mm from the muscle–tendon junction) was selected to maximize the amount of histopathologic changes observed because this area suffers greater injury than the midbelly region. These injury-susceptible regions of the TA were selected to maximize the chance of detecting enough desmin-negative fibers with only 40 stretches to result in significance. The muscle samples were mounted on cork in embedding medium (HistoPrep; Fisher Scientific), frozen in 2-methylbutane cooled by liquid nitrogen, wrapped in aluminum foil, and stored at −80°C.

Immunohistochemistry. Serial sections (8 μm) were cut from the frozen muscle samples in a cryostat at −20°C and stained with azure A to allow a survey of the sample and to assess the degree of histopathologic change. Pathologic myofibers that were hypercontracted, opaque, necrotic, or invaded by mononuclear cells were counted twice in each direction. For more specific identification of injured myofibers, a double-staining technique to localize desmin and laminin in the same section was used.[36] After a blocking step of 5% normal goat serum in phosphate-buffered saline (PBS), pH 7.4, sections were incubated with appropriate dilutions of antibodies at room temperature in a moist chamber. First, mouse anti-desmin immunoglobulin G (IgG; Cat. No. M-0760, Dako Corp, Carpinteria, CA) was applied to the sections in PBS and incubated for 30 minutes. For visualization of desmin, flourescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG (Cat No. F-2266, Sigma) diluted in PBS was applied for 30 minutes. Rabbit anti-laminin IgG (Cat No. L-9393, Sigma) was then added to the same sections and visualized using Cy3-labeled goat anti-rabbit IgG (Cat. No. 111-165-144 Jackson ImmunoResearch Laboratories, West Grove, PA).

Because the laminin antibody was very concentrated, the red color was visible using the FITC filter and allowed for identification of myofibers that were devoid of desmin.[20] The number of desmin-negative myofibers in the entire cross-section of the tibialis anterior was counted manually twice in opposite directions using a magnification objective of 20× and averaged for each sample. For total fiber counting, the microscope slides were placed under an Olympus Provis AX70 microscope and images of laminin-stained muscles were captured using MicroBrightField software (MicroBrightField, Inc., Williston, VT). The images were then imported into Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA). Using a "layer" feature, a dot was placed on each myofiber. The images containing dots were saved as individual data files and were counted using Image ProPlus (Media Cybernetics, Inc., Silver Spring, MD). Injured fibers (i.e., desmin-negative) are presented as percent of total (laminin-positive) fibers.

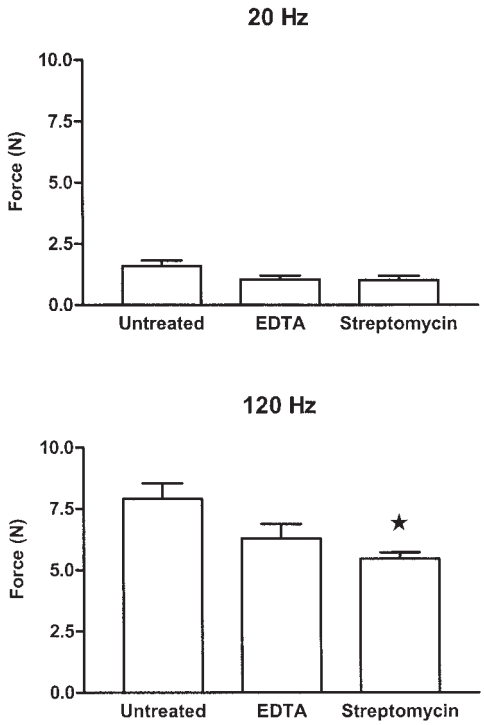

FIGURE 1. Isometric forces at stimulation frequencies of 20 Hz and 120 Hz before the lengthening contractions. Asterisk indicates a significantly lower value for streptomycin-injected rats ($P < 0.05$) than untreated rats.

Statistics. Differences in muscle weights of tibialis anterior muscles, isometric force deficits at 20 Hz and 120 Hz of the dorsiflexor muscle group, and the percent of desmin-negative myofibers were analyzed using a one-way analysis of variance (ANOVA). When a significant *F*-ratio was found, post hoc testing was done with a Bonferroni test to determine where specific differences had occurred. All statistical testing was performed using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA). Values were presented as mean ± SE. Significance was accepted at $P < 0.05$.

RESULTS

Before the lengthening contractions, maximal isometric force of the dorsiflexor muscles with stimula-

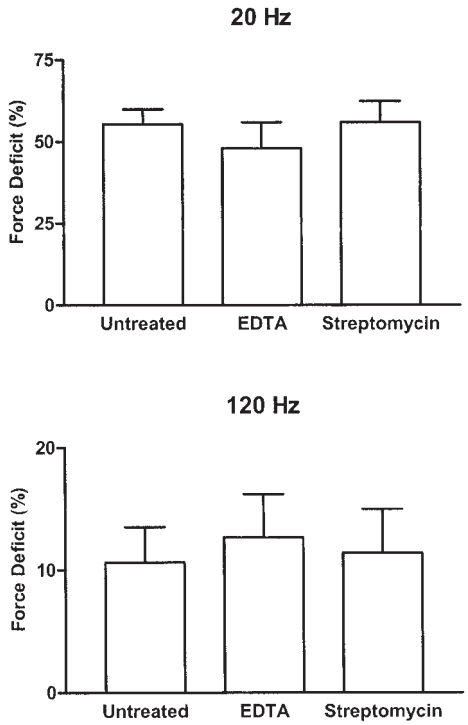

FIGURE 2. Isometric force deficits at stimulation frequencies of 20 Hz and 120 Hz for untreated, EDTA-treated, and streptomycin-treated rats.

tion frequencies of 20 Hz and 120 Hz were measured (Fig. 1). Forces at 20 Hz were similar across all groups. At 120-Hz stimulation, forces were lower for streptomycin-treated rats, but were similar for untreated and EDTA-treated rats.

After the lengthening contractions, the force deficits were substantially lower at 20 Hz than at 120 Hz (Fig. 2). However, there were no differences in isometric force deficits at 20 Hz and 120 Hz between untreated, EDTA-injected, and streptomycin-injected rats (Fig. 2). Thus, the functional outcome induced by the lengthening contractions was similar for all groups.

Two days after the series of lengthening contractions, wet weights of tibialis anterior were increased by the same amount in all injured muscles compared to the contralateral noninjured muscles (C, 8.6 ± 1.3%; EDTA, 7.3 ± 1.0%; S, 8.1 ± 2.2%). However, the number of muscle cells with desmin-negative fibers was significantly lower in streptomycin- and EDTA-treated rats (C, 1.81 ± 0.43%; EDTA, 0.64 ± 0.19%; S, 0.35 ± 0.08%; $P < 0.05$; Fig. 3)—a reduction of 65% (EDTA) and 81% (streptomycin). Likewise, abnormal myofibers identified using azure A staining revealed a similar pattern (C, 1.55 ± 0.40%; EDTA, 0.53 ± 0.17%; S, 0.28 ± 0.08%; $P < 0.05$).

DISCUSSION

In animal models, injury of skeletal muscle from repeated muscle strains is characterized by muscle weakness and delayed appearance of histopathologic changes in muscle fibers.[12,15,17] The histopathologic changes apparently result from an influx of calcium after the lengthening contractions sufficient to activate degradative pathways.[9] To investigate the role of calcium in the cellular response to repeated muscle strains, female Sprague–Dawley rats were treated with streptomycin, an inhibitor of stretch-activated ion channels, or EDTA, a calcium chelator.

Although no difference in the isometric force existed at 20 Hz between groups, maximal isometric force at 120 Hz was reduced in streptomycin-injected rats. This decrease in maximal force could have resulted from a lower muscle weight (10% smaller) of tibialis anterior muscles of streptomycin-injected rats compared to untreated control rats (one-way ANOVA, $P < 0.05$). Nevertheless, the magnitude of the muscle injury (i.e., the force defect at 20 or 120 Hz) was similar for all groups.

Repeated strains, as used in our study, result in sarcomere damage[26] and shearing of T tubules,[30] leading to isometric force deficits (weakness). It appears that sarcomere damage ("popping") and excitation–contraction (E–C) uncoupling occur early in a series of stretches and are linked to the force deficit.[2] Most likely, the initial damage involves overstretched sarcomeres,[24] and because of the attachment of the T tubule to the sarcomeres, T-tubule shearing results. We believe that the greater loss of force at low stimulation frequencies provides indirect evidence for E–C uncoupling. Since the isometric force deficits were not different between groups, sarcomere and T-tubular damage were probably also similar.

No correlation exists for loss of strength (the objective measure of injury) and histopathology.[33] In

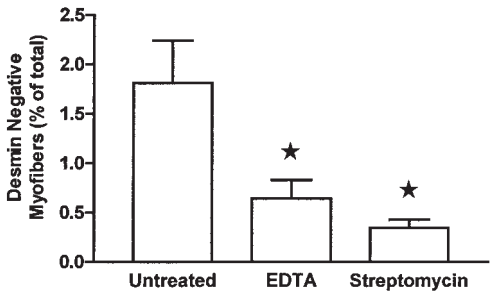

FIGURE 3. Percent of desmin-negative muscle fibers for untreated, EDTA-treated and streptomycin-treated rats. Asterisk indicates significantly lower values ($P < 0.05$) for streptomycin-treated and EDTA-treated rats than for untreated, injured rats.

the present study, isometric force was measured to indicate muscle weakness and desmin-negative fibers in the tibialis anterior muscles were counted to reveal any adverse result of calcium influx. In rats treated with EDTA or streptomycin, a substantial attenuation of desmin-negative cells was observed in spite of a similar loss of isometric strength. These findings support a role for extracellular calcium in the development of histopathologic changes following repeated strains mediated by activation of calcium-activated hydrolases (e.g., calpain) and for stretch-activated ion channels as the mechanism of calcium entry. Similarly, stretch-activated ion channels appear to play a role in the muscle damage seen in muscular dystrophy.[37]

Calcium activation of degradative pathways has been presented for muscle injury from downhill running.[3] In support of this hypothesis, we found that quinacrine, a nonselective inhibitor of the calcium-dependent phospholipase A_2, reduced substantially the histopathologic changes observed after lengthening contractions[36] and the loss of desmin was attenuated when calcium chelation occurred, as demonstrated in the present study. However, treatment with EDTA or streptomycin did not completely prevent histopathologic changes (i.e., presence of necrotic muscle cells; Fig. 3). Since muscle weights increased in all groups, osmotically active substances probably entered some muscle cells by other mechanisms and produced swelling. Skeletal muscles do swell after unaccustomed lengthening contractions.[7,8,13,18] Swelling of the tibialis anterior muscle in all groups after lengthening contractions probably resulted from the influx of other ions (e.g., sodium[38]) or the entry of extracellular proteins, such as albumin,[23,29] resulting from sarcolemmal rupture.

The combination of high strain forces and long muscle length could have been sufficient to cause local membrane damage and influx of a variety of substances. Local membrane damage may have also adversely altered the function of calcium pumps and Na/Ca exchangers[11] necessary to control additional calcium influx. For example, in mice deficient for a Na/Ca exchanger (NCX3), calcium-activated muscle fiber necrosis has been reported.[27] Thus, several pathways may participate in altering intracellular calcium following repeated strains.

In summary, this study illustrates that the mechanisms for muscle weakness and histopathologic changes (inflammation) following repeated muscle strains can largely be dissociated from each other with EDTA or streptomycin treatment. The underlying mechanisms for this dissociation (mechanical damage or calcium influx) help explain why there is no correlation between isometric force deficits and the number of pathologic cells.[33] It also provides a rationale for the observation that the number of pathologic cells continually increases with stretch number but the loss of isometric force appears to reach a fatigue limit.[17] Finally, functional testing in humans exposed to repeated muscle strains, and probably other types of muscle injuries and inflammatory diseases, will not allow the assessment of the degree of muscle pathology.

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