ABSTRACT: Quinacrine is an inhibitor of phospholipase A_2, an enzyme thought to be involved in activity-related injury of skeletal muscles. Histopathologic changes after injury by stretches of activated plantar-flexor muscles were measured in untreated and quinacrine-treated rats. On day 4 of treatment (50 $mg.kg^{-1}$ intraperitoneally for 5 days), 30 stretches were induced by ankle rotation after muscles reached a maximal isometric force. During the stretch protocol, peak stretch forces and isometric force deficits after each stretch [total deficits 56.7 $\pm$ 2.8% (untreated rats) and 59.6 $\pm$ 1.7% (quinacrine-treated rats)] were similar for both groups ($n = 6$ each). Two days after the stretch protocol, histopathologic changes were evaluated using antibody staining on cross-sections of gastrocnemius medialis muscles. Swollen myofibers devoid of desmin were identified. Similar cells, but not all swollen myofibers, in adjacent sections stained for albumin. Quinacrine reduced the number of desmin-negative and albumin-positive cells by 88% ($P < 0.05$) and 84% ($P < 0.05$), indicating that it attenuated histopathologic changes that follow stretch injury of activated skeletal muscles. Histopathologic changes following muscle injury or myopathic disease may thus be reduced or even prevented by selective drug intervention, thereby reducing the risk of muscle fibrosis.

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ATTENUATION OF STRETCH-INDUCED HISTOPATHOLOGIC CHANGES OF SKELETAL MUSCLES BY QUINACRINE

MARK E. T. WILLEMS, PhD, and WILLIAM T. STAUBER, PhD

Department of Physiology and Pharmacology, West Virginia University, P.O. Box 9229, Morgantown, West Virginia 26506-9229, USA

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Histopathologic changes (e.g., swelling of muscle cells, loss of desmin) are common following injury from strenuous, unaccustomed stretches of activated skeletal muscles (i.e., eccentric or lengthening contractions).[12] Such stretches lead to an immediate loss in isometric force production that is the result of excitation/contraction uncoupling and damage to force producing or transmitting structures.[36] Histopathologic changes are most severe after a few days[12,19,26] (e.g., 2 days in rat muscles[3,19]), which is also the case following other muscle injuries such as ischemia/reperfusion, freezing, and blunt trauma.[32] Thus, various injury models, including injury by stretches of activated skeletal muscles, can trigger a pathway that leads to histopathologic changes.[32]

Stretch-induced muscle injury may involve an overload of intracellular calcium if the cell membrane is broken, which subsequently leads to activation of proteases and other enzymes.[1,2] Calcium activation of calpain, a non-lysosomal protease, can degrade desmin,[5] and desmin loss has been reported following stretches of activated muscles.[19,20] Disruption of muscle cell membranes by stretches of activated muscles can also result in intracellular localization of albumin,[23] a protein that is normally excluded from the cell. A key role in the pathway that leads to stretch-induced histopathologic changes induced by calcium influx has been proposed for the enzyme phospholipase A_2 (PLA_2).[1,2] Activation of PLA_2 results in additional membrane damage as well as production of chemoattractants. PLA_2 can be inhibited with quinacrine (mepacrine), an antimalarial drug,[24] and inhibition has been shown to reduce markers of structural injury in various injury models.[7,16,18] For example, following ischemia/reperfusion after coronary artery occlusion, quinacrine reduced the number of necrotic cardiac muscles cells.[7] In addition, in isolated mouse soleus muscle, quinacrine reduced the release of lactate dehydrogenase following repeated isometric contractions.[16] Therefore, quinacrine might protect skeletal muscle from necrosis following stretch-induced injury, although this has never been tested to our knowledge.

Abbreviations: i.p., intraperitoneal; PBS, phosphate-buffered saline; PLA_2, phospholipase A_2

Key words: eccentric contractions; immunohistochemistry; injury; mepacrine; prevention

Correspondence to: M. E. T. Willems; e-mail: mwillems@hsc.wvu.edu

The purpose of the present study was to test the hypothesis that quinacrine would attenuate the amount of histopathologic changes 2 days after injury by stretches of activated skeletal muscles. Histopathologic changes were evident by desmin-negative or albumin-positive cells in cross-sections of the gastrocnemius medialis muscle.

MATERIALS AND METHODS

Animal Care and Preparation. Experiments were performed on female Sprague Dawley rats. All experimental procedures and housing conditions were conducted in accordance with the guidelines of the West Virginia University Animal Care and Use Committee. Use of rats complied with the Animal Welfare Act P.L. 91-579 and guidelines of the Department of Health and Human Services governing the care and use of laboratory animals. Experimenters were not blinded to the treatment condition.

Quinacrine is an inhibitor of PLA_2.[6] A single intraperitoneal (i.p.) injection of 50 $mg.kg^{-1}$ of quinacrine in rats partially prevented myocardial damage by isoproterenol.[31] Pilot experiments revealed that the histopathologic changes were not reduced with three intraperitoneal injections of quinacrine (Sigma Chemical Co., St. Louis, Missouri) in a dose of 50 $mg.kg^{-1}$ (i.e., 1 day before, 1 h before, and 1 day after the stretching). Since rats can tolerate a dose of 50 $mg.kg^{-1}$ of quinacrine for 5 days by intraperitoneal administration, we chose a near-maximal tolerable dose[17] to produce the maximal effect without causing any loss of animals. Six quinacrine-treated rats were tested (mean ± SE for body weight, 223 ± 6 g; age, 109 ± 11 days) and six untreated rats served as controls (body weight, 262 ± 10 g; age 125 ± 11 days). Untreated rats were not injected. Treated rats were injected with quinacrine (50 $mg.kg^{-1}$ i.p.) every day for 5 days (i.e., 3 days before, 1 h before, and 24 h after the stretch protocol).

Rats were anesthetized with brevital sodium (Jones Pharma Inc., St. Louis, Missouri) in an initial dose of 65 $mg.kg^{-1}$ i.p., and supplementary doses were administered to suppress the hindlimb withdrawal reflex to squeezing of the foot. Details on 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.[8,38] Force production of the plantar-flexor muscles was induced by electrical stimulation of the tibial nerve and was recorded as a reaction force under the sole of the foot.[38–40] The force recorded at the sole of the foot is not the actual muscle force that is typically measured at the tendons but the reaction force of the muscles. Brief isometric contractions were used to determine the voltage for maximal force production with a stimulation frequency of 80 Hz. Voltage and pulse duration (200 μs) were kept constant for each muscle preparation. A stimulation frequency of 80 Hz provided near-maximal tetanic force for rat plantar-flexor muscles (Fig. 1). After the functional testing (see below), rats were placed back in their cages with water and food ad libitum until they were sacrificed for tissue sampling.

Stretch Injury Protocol. Repeated stretches (30) of activated plantar-flexor muscles of the left hindlimb were performed by ankle rotations with an angular velocity of $600°.s^{-1}$ from an ankle position of 90° to 40° (ankle position is defined as the angle between the tibia and the plantar surface of the foot, with 180° representing a completely extended foot) with interstretch rest times of 40 s. Each stretch was imposed after a build-up of isometric force for about 400 ms and lasted about 105 ms. All stretches were performed with a stimulation frequency of 80 Hz and a stimulation time of 900 ms; muscles from untreated and quinacrine-treated rats produce near-maximal isometric force at this frequency (Fig. 1). To examine the isometric force loss by the 30th contraction, an isometric contraction of 600 ms was performed 40 s after the last stretch.

Force–Frequency Measurements. In all groups, before and after 1 h of rest following the repeated stretches, force–frequency measurements were performed at an ankle position of 90°. Muscles were stimulated at frequencies of between 5 and 100 Hz. Different train durations, i.e., for 1,500 ms (5, 10,

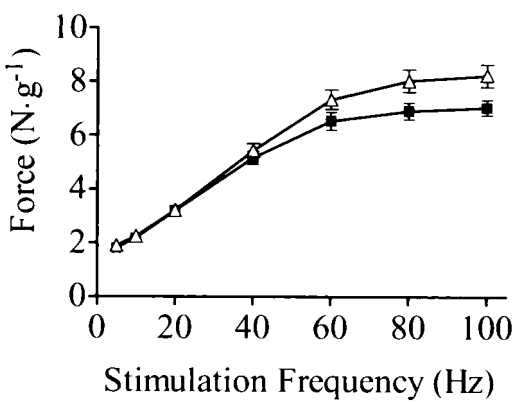

FIGURE 1. Force–frequency relationships of rat plantar-flexor muscles. Isometric force was normalized to muscle mass of the plantar-flexor muscles. Open triangles, untreated animals; filled squares, quinacrine-treated animals (mean ± SE).

and 20 Hz), 900 ms (40 Hz), and 600 ms (60, 80, and 100 Hz) were used to guarantee the recording of near-maximal or maximal force at each stimulation frequency and to minimize fatigue. The isometric force deficits following recovery for 1 h were taken as indirect evidence of stretch-induced muscle injury[4] because 1 h of rest is sufficient to allow the rat plantar-flexor muscles to recover from fatigue by contractions with high stimulation frequency.[39]

Muscle Sampling. In pilot studies using the medial gastrocnemius muscle, histopathologic changes were evident at 24 h after similar stretch protocols, reaching a peak at 48 h and becoming less with each subsequent day. Plantar-flexor muscles (soleus, plantaris, and gastrocnemius muscles) from the left (stretched) and right (unstretched) hindlimb were excised 2 days after the stretch protocol to maximize the probability of observing histopathologic changes. All muscles were trimmed and blotted, and wet weights were measured on a balance. The wet weights of the plantar-flexor muscles were not different between left and right hindlimb (data not shown).

Transverse sections from the distal part of the gastrocnemius medialis muscles (5–10 mm from the muscle–tendon junction) were mounted on cork in embedding medium (HistoPrep; Fisher Scientific, Fair Lawn, New Jersey). Sections were frozen in 2-methylbutane cooled by liquid nitrogen, wrapped in aluminum foil, and stored at −80°C until histochemical analysis.

Immunohistochemistry. Serial sections (8 μm) were cut in a cryostat at −20°C. The following antibodies were used for identification of specific proteins on sectioned muscle samples: (1) desmin (Cat. No. M0760, Dako Corp., Carpinteria, California) to evaluate the cytoskeleton,[20] (2) laminin (Cat. No. L9393, Sigma) to visualize the basal lamina of muscle cells,[13] and (3) albumin (Cat. No. W90341C, Biodesign International, Kennebunk, Maine) to define injured muscle cells.[23] Six sections per animal were analyzed for each antibody. Desmin and laminin were applied as a double-label protocol similar to the fluorescence method described previously for desmin and dystrophin.[30] In brief, after a brief wash in phosphate-buffered saline (PBS, pH 7.4), a solution of normal goat serum (5% in PBS) was applied to the slides and allowed to incubate for 30 min at room temperature in a moist chamber. After a 10-min wash, a 40-μl aliquot of mouse antidesmin IgG (20× dilution) was applied to the slides and the slides were incubated for 30 min at room temperature. After a second wash, a 40-μl aliquot of fluorescein-labeled sheep anti-mouse IgG (20× dilution, Cat. No. F2266, Sigma) was applied and again incubated for 30 min at room temperature. After a third wash, 40 μl of rabbit antilaminin IgG (200× dilution) was applied and incubated for 30 min at room temperature. After a fourth wash, a 40-μl aliquot of Cy3-labeled goat anti-rabbit IgG (500× dilution, Cat. No. 111-165-144, Jackson Immunoresearch Labs, West Grove, Pennsylvania) was applied and incubated for 30 min at room temperature. After a final wash in PBS, coverslips were applied to the slides.

The slides were viewed under a fluorescence microscope and 35-mm photographs were taken of areas of interest. Because the laminin antibody was concentrated, the red color was visible using the FITC filter and allowed identification of myofibers that were devoid of desmin.[21] Albumin was localized using a single-label protocol, i.e., one primary antibody (100× dilution) was applied to the tissue section.[28] No abnormal muscle cells were observed in soleus and plantaris muscles. Using a fluorescence microscope with a magnification objective of 20×, the number of desmin-negative and albumin-positive fibers in the entire cross-sections of gastrocnemius medialis muscles were counted manually twice, in opposite directions from separate but adjacent sections, and averaged for each section. Quantitation of desmin-negative or albumin-positive cells was performed independently of each other. Using our method of desmin localization, not all desmin staining was uniform, as desmin is located in the region of the Z-line, so variations can occur from myofiber to myofiber due to the plane of the section; variations in staining were not seen with laminin. We were very conservative in our counting and, to be sure that desmin was present in faintly stained myofibers and absent in dark myofibers, we observed sections at higher magnifications where the staining is much brighter.

Statistics. Changes in peak stretch force (i.e., the sum of active and passive force at the end of the stretch phase) and isometric force deficits were analyzed by using treatment (quinacrine and untreated) by contraction number (1, 2, 30) ANOVAs with repeated measures on the contraction factor. Analysis of the isometric force deficits 1 h after the stretches was conducted by using treatment (quinacrine and untreated) by stimulation frequency (5, 10, 20, 40, 60, 80, 100 Hz) ANOVAs with repeated measures on the frequency factor.

When a significant F-ratio was found, post-hoc testing was done with a Bonferroni test to determine where specific differences had occurred. A Student's *t*-test was performed to test for desmin-negative and

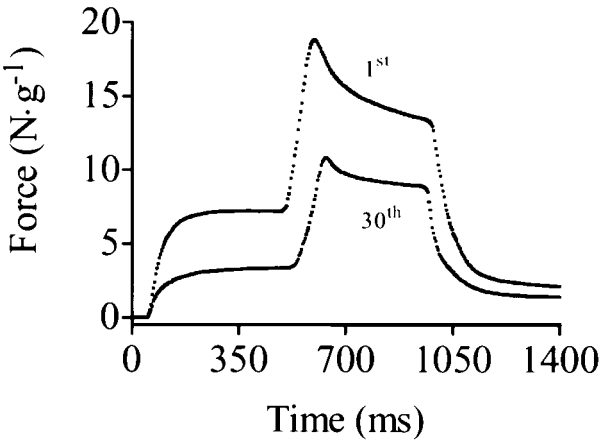

FIGURE 2. Examples of the force of rat plantar-flexor muscles for the 1st and 30th contraction in a series of stretches from an untreated rat. Force was normalized to muscle mass of the plantar-flexor muscles.

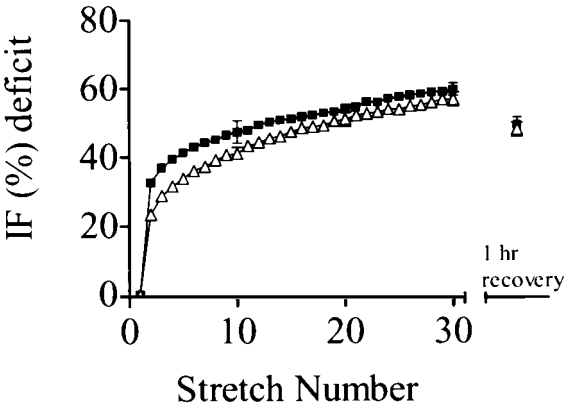

FIGURE 4. Isometric force (IF) deficits (mean ± SE) at an ankle position of 90° as a function of stretch number and after 1 h of rest. Open triangles, untreated animals; filled squares, quinacrine-treated animals. For clarity of the figure, only error bars of contractions 10, 20, and 30, and after 1 h of rest are plotted.

albumin-positive fibers. All statistical testing was performed by using Graphpad Prism version 3.00 (Graphpad Software, San Diego, California). Values are presented as mean ± SE. Significance is accepted at $P < 0.05$.

RESULTS

Force Measurements. No adverse effects of short-term treatment with quinacrine on force production were present. Force–frequency relationships of plantar-flexor muscles of untreated and quinacrine-treated rats were similar (Fig. 1). In Figure 2, typical examples of forces produced during the 1st and 30th contractions with stretches are illustrated. All contractions reached an isometric plateau phase within 150–200 ms after onset of stimulation. Peak stretch forces were similar for the first contraction (untreated, 17.7 ± 1.1 N/g; quinacrine-treated, 19.0 ± 1.3 N/g). Peak stretch forces decreased during the stretch protocols but were similar for untreated and quinacrine-treated rats at comparable contraction numbers (Fig. 3). Repeated stretches resulted in similar isometric force deficits for untreated and quinacrine-treated rats. Deficits in isometric force immediately after the last (30th) contraction were 56.7 ± 2.8% for untreated and 59.6 ± 1.7% for quinacrine-treated rats. After 1 h of rest, recovery of the force value of the 30th contraction (stimulation frequency, 80 Hz) was 21.0 ± 3.1% and 24.5 ± 3.3% for untreated and quinacrine-treated rats, respectively (Fig. 4), and this difference was not significant.

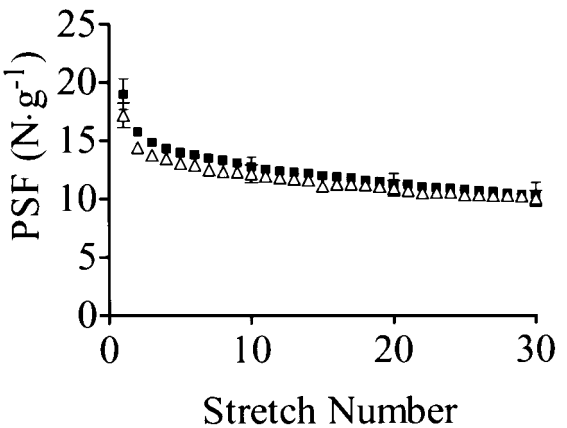

FIGURE 3. Peak stretch force (PSF) (mean ± SE) at an ankle position of 40° as a function of stretch number. Open triangles, untreated animals; filled squares, quinacrine-treated animals. Peak stretch force was normalized to muscle mass of the plantar-flexor muscles. For clarity, only error bars of contractions 1, 10, 20, and 30 are plotted.

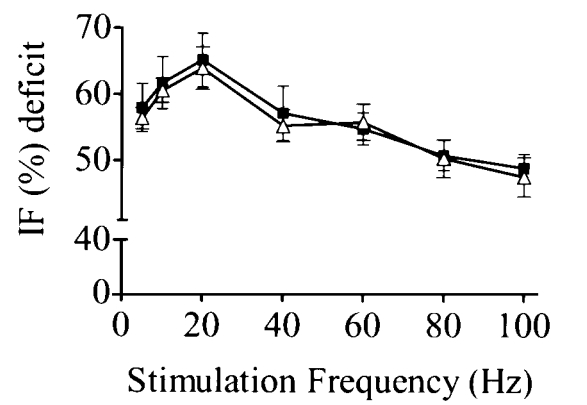

FIGURE 5. Relationship between stimulation frequency and the isometric force (IF) deficits (mean ± SE) at an ankle position of 90° after 1 h of rest following the stretches. Open triangles, untreated animals; filled squares, quinacrine-treated animals.

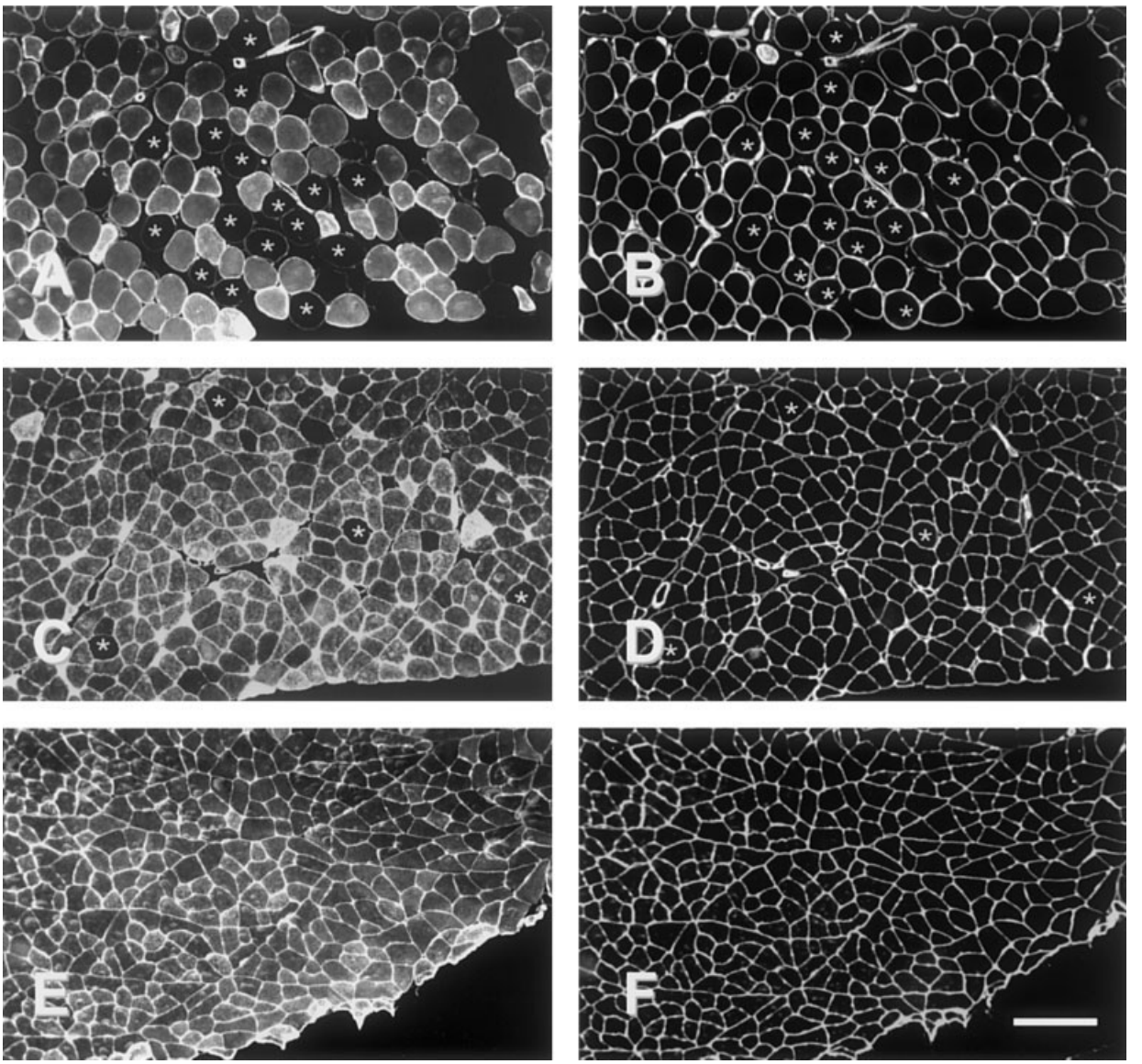

FIGURE 6. Representative photomicrographs of the region of the gastrocnemius medialis muscle in which pathologic muscle cells were observed after stretches in untreated rats **(A, B)** and quinacrine-treated rats **(C, D).** Panels **(E)** and **(F)** are from uninjured muscles in untreated rats. Left panels **(A, C, E)** show desmin, and right panels **(B, D, F)** show laminin co-localization. Asterisks indicate muscle cells that were devoid of desmin but which stained positive for laminin. Bar = 200 μm.

After 1 h of rest, substantial force deficits were present at all stimulation frequencies but there were no differences for isometric force deficits at each stimulation frequency between untreated and quinacrine-treated rats (Fig. 5).

Histopathologic Changes after 2 Days. Histopathologic changes were identified by swollen (rounded) myofibers that stained for albumin and laminin but were devoid of desmin (Fig. 6). All desmin-negative or albumin-positive cells stained for fast myosin.[29] Histopathologic changes were substantially reduced in quinacrine-treated rats compared to untreated rats. In quinacrine-treated rats, the number of desmin-negative and albumin-positive fibers were 88% ($P < 0.05$) and 84% ($P < 0.05$) lower, respectively, compared to untreated rats.

DISCUSSION

Skeletal muscle injury by repeated stretches of activated skeletal muscles results in an immediate force deficit[15,34,40] and is often accompanied by the delayed onset of histopathologic changes indicative of injured myofibers. Those studies that have measured isometric force deficits and markers of muscle dam-

age at the same time-point have found a poor correlation between them.[35] The magnitude of the force deficit depends on the magnitude of the peak stretch force,[34] which corresponds generally to the amount of injured myofibers.[22] Since treatment of rats with quinacrine did not reduce the peak stretch force compared to untreated rats, the injurious stimulus was presumed to be identical for the two groups of animals.

Two days following repeated strain injury, histopathologic changes become readily apparent and are thought to be mediated by calcium-overload producing cell death and fiber necrosis.[1,2] Although intracellular calcium levels were not measured, the loss of desmin, a protein of the cytoskeleton that is susceptible to degradation by calpains,[14] and the entry of albumin into the myofibers (due to a loss in membrane integrity) provide indirect evidence that extracellular calcium may have entered the cells. Elevations in intracellular calcium would activate both calpains and phospholipases,[2,5] which could further injure cellular membranes.

Activation of PLA_2[1,10,16] by elevated levels of intracellular calcium results in the production of eicosanoids that act as potent inflammatory mediators and are implicated in the development of histopathologic changes.[2] Inhibition of cellular damage in skeletal and cardiac muscle by quinacrine, a putative PLA_2 inhibitor, has been reported.[7,16] However, quinacrine may have blocked the entry of calcium into muscle cells by other membrane-specific actions.[6,9,11,27] Nevertheless, attenuation of histopathologic changes with quinacrine treatment implies that calcium homeostasis was maintained in most muscle cells.

The present study has demonstrated that quinacrine treatment attenuates histopathologic changes resulting from stretch-induced muscle injury of activated skeletal muscles. Since dystrophic muscles[26,37] and muscles from unloading[33] or spaceflight[25] are more sensitive to injury, specific phospholipase inhibition might likewise reduce the severity of other muscle injuries. However, quinacrine may have exerted its protective effect not only by a direct inhibition of PLA_2 activity, but also by blocking calcium entry following repeated stretches. Regardless, our findings suggest that histopathologic changes following muscle injury or myopathic disease may be reduced or even prevented by selective drug intervention, thereby reducing the risk of muscle fibrosis.

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