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Disruptions of Muscle Fiber Plasma Membranes

Role in Exercise-induced Damage

Paul L. McNeil and Robert Khakee
From the Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts

The authors have tested the hypothesis that plasma membrane disruptions are an early form of structural damage to the fibers of eccentrically exercised muscle. Rat serum albumin (RSA) was used as a marker for muscle-fiber wounding in the rat tricep (medial head) exercised eccentrically by downhill running. In all muscles examined, strong staining with a horseradish peroxidase (HRP)-conjugated anti-RSA antibody was observed between fibers (intercellular staining) and also within certain fibers (intracellular staining). This intracellular staining was interpreted as identifying muscle fibers wounded at their plasma membranes and hence rendered transiently or permanently permeable to extracellular RSA. The most striking finding of this study was a 6.9-fold increase relative to unexercised controls in the number of wounded cells in the medial head immediately after eccentric exercise. The authors also reproducibly observed, albeit less frequently, myocytes that stained with anti-RSA in the medial head and several other muscles of the "unexercised," caged laboratory rat. The extreme vulnerability of muscle plasma membranes to mechanically induced stress was revealed in this study by HRP injections into the triceps long head. A single injection of 200 μl HRP through a 26-gauge needle resulted in extensive labeling of the muscle fibers present in the long head cross-sectioned at the injection site. The authors propose that initially resealable and/or highly localized, unsealable membrane wounds are an early form of exercise-induced damage that could progress along the length of the fiber until, 1 to 4 days after eccentric exercise, it becomes sufficiently severe that it can be readily recognized as the frank fiber necrosis and cellular infiltration described in numerous previous studies. In possessing cells wounded at their plasma membranes, normal, undisturbed rat muscle and eccentrically exercised muscle appears to resemble gut and skin, two additional tissues routinely exposed to mechanical forces** in vivo. ***The authors propose that membrane disruptions provide a route into and out of myofiber cytoplasm distinct from the conventional, membrane-bounded routes of endo- and exocytosis, and therefore may be of importance both technically, as a route for introducing foreign genes into muscle cells, and biologically, as a route for release of the growth factor, basic fibroblast growth factor. (Am J Pathol 1992, 140:1097–1109)

Muscles can be damaged by exercise.[1] Particularly striking and well studied is the muscle damage induced by eccentric contractions, in which the body of the muscle is lengthened while its constituent fibers are activated.[2] Downhill running in humans and rodents, for example, causes eccentric contraction of certain leg muscles and fiber damage.

Muscle damage that is detectable by conventional histologic techniques is not, however, generally seen until 2 to 4 days after eccentric exercise, when frank fiber destruction or infiltration of inflammatory white cells has been described in numerous models.[3–5] Similarly, serum levels of creatine phosphokinase (CK), an enzyme believed to be released from injured or dying muscle cells, generally do not peak until 1 to 14 days after eccentric exercise.[3,6–9] However, immmediately after eccentric exercise there is in some models a loss of the exercised muscle's capacity for force production,[7] a transient rise in serum CK levels,[10,11] and fiber damage detectable in thin plastic sections examined by light microscopy[12] or ultrathin sections examined by electron microscopy.[13] These latter results strongly suggest that an early injury of some kind does occur, and support a model in which

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Address reprint requests to Dr. Paul L. McNeil, Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115.

some form of early structural damage, not readily visualized or quantitated by conventional histologic methods, leads to immediate loss of muscle force generating capacity. If not repairable, this damage then progresses to fiber degeneration and death so readily demonstrable by standard histologic or enzymatic assay 1 to 4 days after muscle exercise.

We propose that plasma membrane disruptions are an early form of structural damage to muscle fibers of eccentrically exercised muscle. To test this hypothesis, we have applied previously developed immunohistochemical methods for detecting cells wounded at their plasma membranes *in vivo*[14,15] to the medial head of the triceps of rats exercised by downhill running.[10] We provide qualitative and quantitative evidence that the plasma membranes of fibers in this muscle are wounded far more frequently than those of the medial triceps of the unexercised rat. We also, however, provide evidence that a chronic level of membrane wounding occurs in the muscles of the caged, "unexercised" laboratory rat, and that muscle fibers are vulnerable to certain forms of mechanical injury. We suggest that plasma membrane wounding is a common but previously undetected event in the life of the muscle cell.

Materials and Methods

Animals

Male Sprague Dawley rats of various weights and ages were tested, and 350-g to 400-g animals seemed to be best suited for running the 90-minute period. The rats were housed in an animal facility until 1 to 2 days before each experiment. This short period in the laboratory afforded the rats time to adjust to their surroundings and made them better running animals. Of the four rats that were placed on the treadmill at the beginning of each experiment, two or three rats usually completed the run.

Running Experiment

Rats were run continuously at a constant speed of 16 m/min for 90 minutes on an Ohio Gear treadmill (loaned by C. R. Taylor, Harvard University) at an incline of 16° downhill.[10] An electrified grid, placed at the top of the treadmill and under manual control, was activated sparingly to enforce exercise. In addition to electrical shock, hand prodding was sometimes used.

In Situ *Fixation of Muscle*

Upon completion of exercise, the experimental animals were either allowed 24 hours to recover from the physical stress (24-hour runner) or they were perfused immediately after running (0-hour runner). Each rat was exposed to chloroform inhalation for a short period followed by an intraperitoneal injection of Nembutol (Abbot Laboratories, North Chicago, IL) to anesthetize the animal. The heart was thoroughly exposed by standard surgical procedures and 0.2 ml heparin (10,000 U/ml) was introduced into the left ventricle. A sample of blood was taken from the left ventricle for plasma enzyme analysis. Next, a small incision was made in the left ventricle, a cannula was passed up through the heart into the base of the aorta, and 500 ml of 0.1% procaine in phosphate-buffered saline (PBS) (37 C) was perfused. The animal was then perfused with 250 ml of freshly prepared 8% paraformaldehyde (37 C) in calcium and magnesium free PBS (CMF-PBS). One hour after completion of perfusion, the entire forelimb was skinned, amputated, and immersed for further fixation for 24 to 48 hours in paraformaldehyde. Paraformaldehyde was chosen as a fixative because it preserved the antigenicity of RSA and because it did not significantly increase muscle background fluorescence (autofluorescence). Glutaraldehyde was unsatisfactory in both of these respects and thus unsuitable for either rat serum albumin (RSA) or lysine-conjugated fluorescein dextran (FDxLys) labeling of wounded muscle fibers. Formalin has previously been used to fix FDxLys (e.g. 17). The triceps muscle was exposed, divided into its respective muscle groups, and placed into 30% sucrose solution until frozen sectioning, usually 2 days later.

Histochemical Analysis

Each muscle bundle was trimmed and mounted for cross-sectional analysis. Muscle bundles to be compared quantitatively were mounted side by side and sectioned simultaneously on the same block so as to ensure equivalency of specimen thickness and postsectioning processing (i.e. antibody staining). The muscle was embedded in Tissue Tek OCT (Miles Scientific, Naperville, IL) and frozen in isopentane cooled with liquid nitrogen. Sections of 12 μm to 20 μm thickness were cut at −22 C in a Hacker Bright microtome cryostat and picked up onto gelatin-coated slides. The sections were immediately immersed for further fixation in 8% (wt/vol) paraformaldehyde for 10 minutes, washed twice in PBS, and incubated for 10 minutes in 10 mmol/l ammonium chloride in PBS. After three further washes in PBS, the sections were permeabilized in 0.2% (wt/vol) Triton X-100 in PBS for 10 minutes. Next the slides were placed directly into blocking buffer that contained 4% (wt/vol) heat-inactivated sheep serum (Sigma Chemical Company, St. Louis, MO) and 0.05% Triton X-100 for 1 hour, with one change of solution. The slides were then incubated in a

1:400 dilution of horseradish peroxidase (HRP) conjugated IgG fraction sheep anti-rat albumin (Cappel Research Products, Durham, NC) in blocking buffer at 37 C overnight. The following morning, sections were reacted with diaminobenzadine (DAB) intensified with cobalt chloride.[16] The muscle sections were mounted in deionized water for light microscopic examination.

Muscle Injection Experiments

After anesthetization as aforementioned, the triceps or quadriceps muscle of each animal was exposed. Animals received a 0.2-ml injection through a 26-gauge needle of carbon black in PBS containing HRP (40 mg/ml), FDxLys (20 mg/ml), or no additions, and were allowed to recover for 2 or 24 hours before sacrifice as outlined earlier. After perfusion-fixation and sucrose cryoprotection, as mentioned earlier, 12 μm to 20 μm frozen sections were cut and subjected to a DAB reaction or histochemical analysis as outlined previously.

Plasma Enzyme Analysis

Creatine phosphokinase analysis was performed on the serum samples that were collected before perfusion of each animal. The blood serum was frozen immediately after centrifugation for 4 to 7 days before analysis. CK-mediated reduction of $NADP^+$ was measured spectrophotometrically at 340 nm and 25 C as outlined in the manufacturer's protocol (Sigma Diagnostics Creatine Phosphokinase kit). The total number of 0-hour, 24-hour, and control animals assessed were 8, 4, and 8, respectively.

Image Analysis

The immunostained muscle sections were viewed by light microscopy to determine if the staining in serial sections was consistent and to ensure that no serious sectioning artifacts (folds, tears, etc.) were present. Images were acquired from a Zeiss Photomicroscope III (Carl Zeiss, Oberkochen, West Germany) through a Dage-MTI (Dage MTI Inc., Michigan City, IN) or a Hamamatsu camera (Hammamatsu Systems Inc., Waltham, MA) on a Tracor Northern 8500 Image analysis computer system (Tracor Inc., Middleton, WI). Images were acquired only when the microscope stage was positioned at its 1-mm hash marks, ensuring random collection of data for quantitative analysis. Edges of the sections were avoided, due to damage incurred there during muscle dissection and subsequent processing. Microscope illumination intensity, and video camera black and gain levels, remained constant for analysis of all muscle sections. Fifty frames were acquired per image and digitized to yield light intensity levels ranging in shades of gray from 1 (black) to 255 (white). A binary template was positioned using a mouse controller on the central portion of the digitized image of each myofiber. This initially 1-pixel template was then expanded to an 11 × 11 (121) pixel box. A histogram was generated of the number of pixels illuminated within each pixel box at the various intensity levels (1–255) and this was printed as a hard copy for further analysis. The triceps of six 0-hour runners, two 24-hour runners, and six control, unexercised animals were analyzed. The total number of muscle fibers analyzed in each group were: 0-hour runner, 9151 fibers; 24-hour runner, 4245 fibers; and unexercised, control, 8725 fibers. Approximately 19% of the total number of fibers present in each triceps cross-section was analyzed.

Fluorescein Dextran

After anesthetization as previously described, each experimental animal was intravenously injected with 0.5 ml of PBS containing 400 mg/ml lysine fixable fluorescein dextran (FDxLys) (~10,000 mol. wt.) synthesized as described.[17] Twenty-four h later, injected animals and uninjected control animals were perfusion-fixed and the triceps muscles were sectioned as described above at thicknesses of 10–20 μm. Sections were observed immediately by fluorescence microscopy following mounting in p-phenylenediamine, an anti-bleaching mounting agent.[18]

Results

Anti-RSA Staining

Microscopic Observations

RSA was used as described previously in studies of skin[15] as a marker for muscle fiber wounding in the rat tricep (medial head) exercised eccentrically by downhill running.[10] In all muscles examined, strong staining with an HRP-conjugated anti-RSA antibody was observed between fibers (intercellular staining) and also within certain fibers (intracellular staining) (Figure 1). This staining was specific for RSA since it could be completely eliminated by a preincubation of the anti-RSA with a 40-fold molar excess of RSA (not shown). The intracellular staining was interpreted as identifying muscle fibers wounded at their plasma membranes and hence rendered transiently or permanently permeable to extracellular RSA, as previously documented.[14,15] Usually, this intracellular staining was diffusely distributed across the sarcoplasm, al-

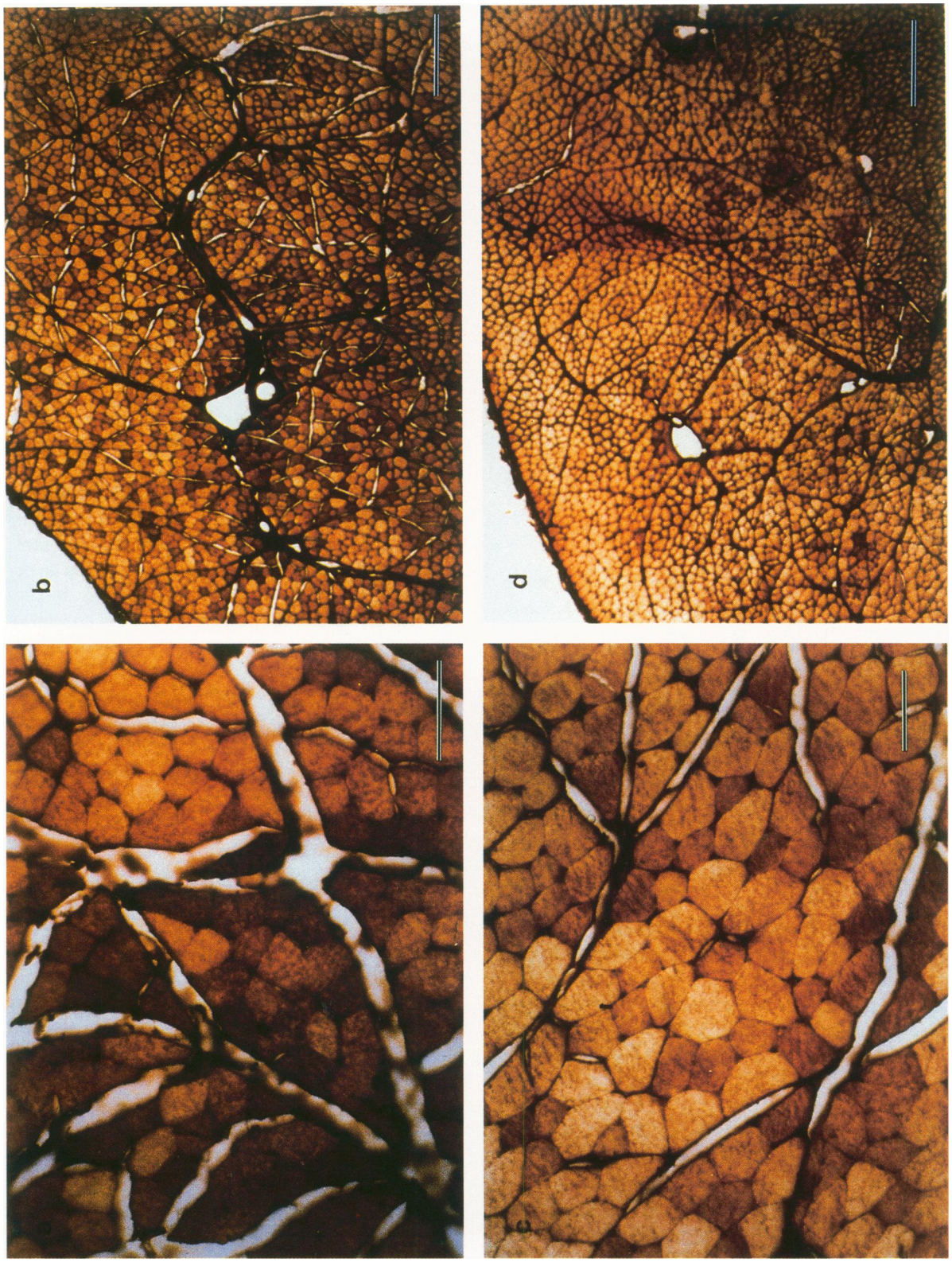
b
d

Figure 1. *Photomicrographs of medial heads of the rat tricep stained with HRP-conjugated anti-RSA.* **a, b**: *A 0-hour medial head, taken from a rat immediately after downhill running.* **c, d**: *A control medial head taken from an unexercised rat. Bars:* **(a, c)** = *20 μm;* **(b,d)** = *500 μm.*

though sometimes it appeared to be localized to the fiber periphery. Examination of serial sections showed that the pattern of intracellular staining was constant from one section to another (not shown).

Microscopic comparison of medial heads obtained immediately after downhill running (0-hour runner; Figure 1a, b) with unexercised (control; Figure 1c, d) medial heads clearly suggested that eccentric exercise of the muscle had increased the number of intracellularly stained fibers. No obvious difference of this kind was observed between medial heads obtained 24 hours after exercise (24-hour runner) (not shown) and control medial heads. Intracellular staining of some fibers was, however, seen in both the control and 24-hour medial heads, suggesting the need for a quantitative analysis of the frequency of fiber staining as a function of eccentric exercise. This "low" level of intracellular fiber staining was a constant feature of the muscles that we examined from the unexercised rat, including the long and lateral heads of the tricep and the gastrocnemius muscles (not shown).

Quantitation

Randomly acquired video images of control and exercised medial heads were digitized and quantitatively analyzed using a Tracor image analysis system. In brief, the intensity value of all pixels contained within all square zones placed at the center of each fiber in the digitized image (Figure 2a, c, e) were selected and recorded into computer memory. These values were then plotted as a hard copy for ease of analysis (Figure 2b,d,f). A threshold value was determined by selecting through the microscope eyepieces fibers that, subjectively, were judged to be stained intracellularly with the anti-RSA, and then obtaining the average pixel value registered in a standard measurement of that fiber. Fibers with pixel values falling below this threshold were scored as positively stained.

Positively stained fibers thus identified were more numerous (3.9- to 690.3-fold) in each of the six triceps (six separate rats) from 0-hour runners than in those of the paired (i.e., cut on the same block, picked up onto the same slide for immunostaining, etc.; also from six separate rats) controls (Figure 3a). The mean number of positively stained fibers in the entire 0-hour population was 6.9-fold higher than in the entire control population (Figure 3b). Our measurements, however, showed no significant difference between control and 24-hour medial heads. Twenty-one percent of the fibers were classified as intracellularly stained in 0-hour medial heads taken immediately after exercise, compared with 3.13% in control and 5.63% in 24-hour medial heads (Figure 3c). Thus, our quantitative data confirm that eccentric exercise of the medial head increases anti-RSA staining, which we interpret as identifying myofibers wounded at their plasma membranes.

Fiber Wounding Induced by Experimentally Imposed Mechanical Force

To examine the vulnerability of muscle fiber membranes to mechanical force and to validate RSA as a label for muscle fiber plasma membrane wounding, we punctured the rat triceps (long head) with a 26-guage needle and then injected either 200 μl of HRP (40 mg/ml), FDxLys (20 mg/ml), or saline alone. HRP and FDxLys have previously been used as markers for cell wounding.[14,15] HRP injection into the long head of the rat tricep resulted in a strikingly extensive labeling of fibers throughout the diameter of the long head processed 2 or 3 hours after the injection (Figure 4a). The injection site was surrounded by a rim of particularly heavily labeled fibers. Extensive labeling was still evident 24 hours after the injection, when less intercellular HRP was present, suggesting trapping of the HRP in fibers that successfully resealed plasma membrane wounds (Figure 4b). Injections with FDxLys similarly resulted in a large number of labeled fibers at 2 and 24 hours after the injection (not shown). Moreover, injections of the quadriceps with either probe resulted in similarly striking labeling of the fibers of this muscle (not shown). When serial sections of the HRP-injected muscles were stained in addition with HRP-coupled anti-RSA, the frequency and intensity of labeling was dramatically increased, as would be expected if RSA and HRP were both acting as labels of wounded fibers (Figure 4c). Finally, in triceps injected with saline alone extensive fiber labeling with anti-RSA (coupled to HRP or FITC) was detected and was particularly prominent around the injection site, both at 3 (Figure 5) and 24 hours (Figure 6) postinjection.

Conventional Histology and CK Levels

Parafin-embedded medial heads of control, 0-hour, 24-hour, and 48-hour animals were cut at 8 μm, and the sections stained with hematoxylin and eosin. Overt damage to muscle fibers, such as fiber necrosis and infiltration with fibroblasts, monocytes, and macrophages, was seen only in the 48-hour specimen, and there involved less than 1% of the fibers (not shown). By contrast, fiber necrosis along the edge of a needle puncture wound was readily observed in muscle taken 24 hours post-

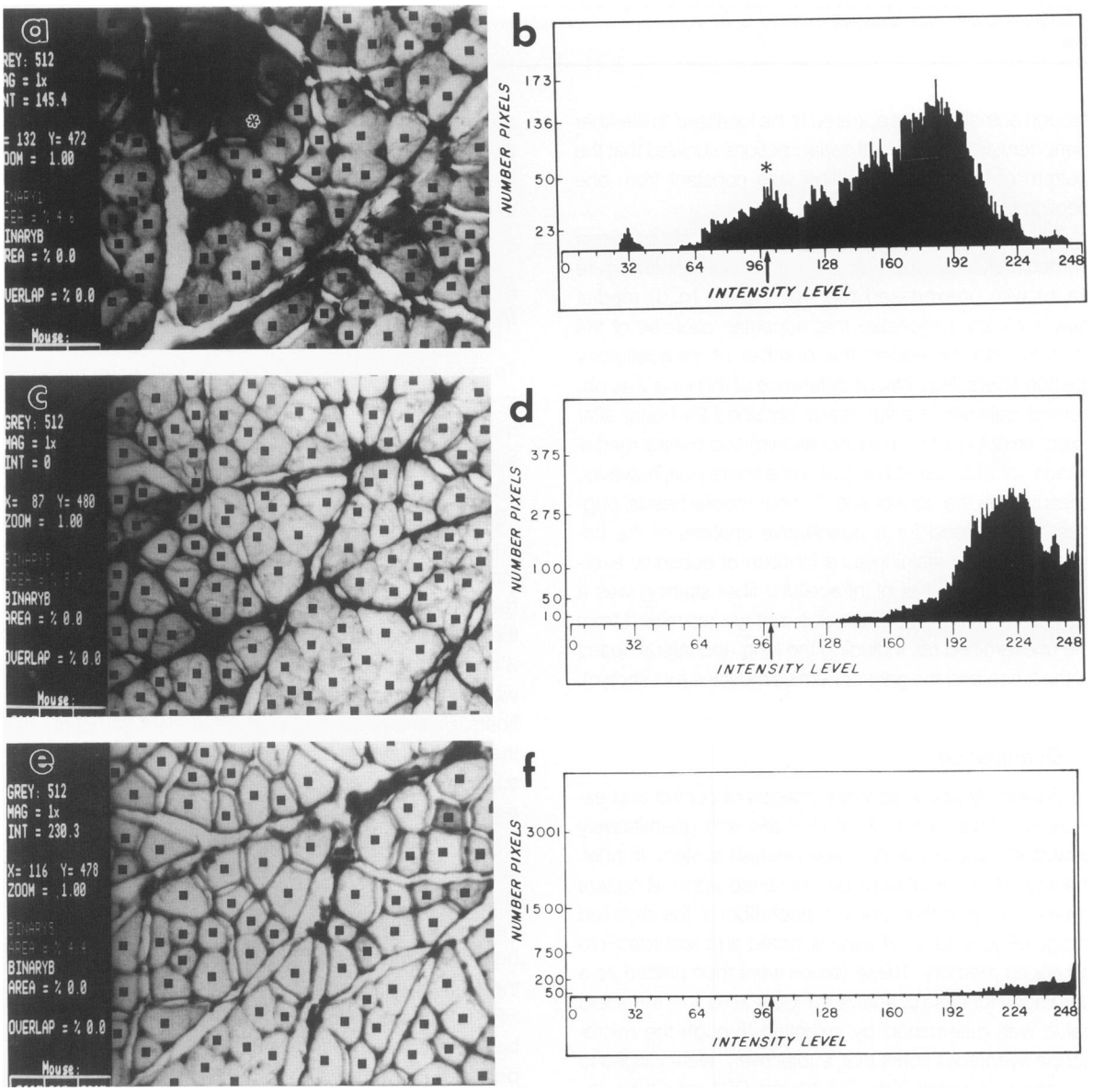

Figure 2. *Photographs of the digitized, video images of medial heads stained with anti-RSA from 0 hour* **(a)**, *24 hour* **(c)**, *and control* **(e)** *rats and corresponding histogram plots of the number of pixels at the various intensity levels (1–255) resulting from analysis of each image* **(b, d, f)**. *Note the binary templates (squares) at the center of each myofiber in the photomicrographs from which the pixel values plotted in the corresponding histograms were obtained. Note also the pixel intensity threshold of 100 (arrow) on the histograms, below which pixel values were defined as indicating labeling with anti-RSA. An asterisk in* **(a)** *marks a labeled fiber and in* **(b)** *its corresponding position (average pixel value) on the histogram.*

puncture (not shown). A significant elevation relative to control animals of serum CK levels was measured in 0-hour animals, but not of 24-hour animals, consistent with previous CK measurements in this model (Figure 7).[10]

FDxLys Staining

As an alternative to RSA as a label for fiber wounding, we injected FDxLys (10,000 mol wt) intravenously. In long heads of injected, unexercised animals, we observed frequent intracellular labeling of fibers with this fluorescent, normally membrane impermeant molecule (Figure 8a), as is indicated here by comparison with the fluorescence image of a long head from a control, uninjected rat (Figure 8b). This provides independent evidence that muscle fiber wounding occurs even in the unexercised rat.

Discussion

Rat muscle fibers were classified as wounded at their plasma membrane if they contained a normally mem-

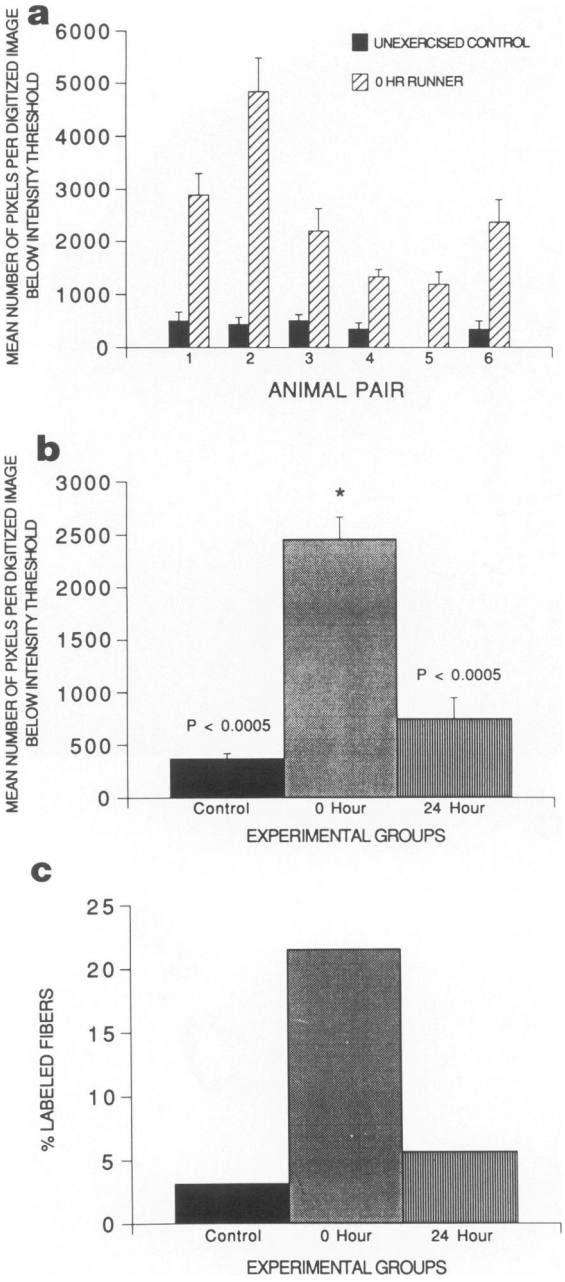

Figure 3. *Quantitative image analysis of anti-RSA labeling of medial heads.* **a**: *Mean number of pixels per digitized image falling below the threshold indicative of anti-RSA labeling for each of six animal pairs. Each pair of triceps, 0-hour runner and unexercised control, was cut on the same block, and picked up on the same slide to insure equality of section thickness and post-sectioning processing. The number of images (n) examined for each pair were: 1) 15, 15 (control, 0 h); 2) 16, 16; 3) 21, 21; 4) 13, 18; 5) 14, 14; and 6) 11, 13.* **b**: *Mean derived from the entire rat population of the number of pixels per digitized image falling below the threshold defined as indicative of anti-RSA labeling. The number of digitized images examined (n) in the 0-hour, 24-hour and control experiments were, respectively, 97, 39, and 90. Bars indicate SEM, and P values relative to the 0-hour animal (asterisk) are shown.* **c**: *The percentage of fibers labeled with anti-RSA in the control, 0-hour and 24-hour medial head rat populations* (**b**).

brane impermeant "tracer" molecule in their cytoplasm. We have used predominantly the rat's own albumin as a tracer for myocyte wounding. As an endogenous protein that normally bathes the myocytes at a high concentration, RSA avoids two disadvantages of an exogenous tracer: the presence in tissue of a high concentration of (a) a "foreign" molecule that (b) has to be injected either directly into the tissue (Figure 4) or into the blood stream.

The main disadvantage of RSA arises from its high extracellular concentration around the fiber. Most, but not all, of this "background" of extracellular RSA could be washed away by the 500-ml saline perfusion before fixation. The concern therefore is that any observed fiber labeling is an artifact of leakage of incompletely fixed RSA into fibers during tissue processing, rather than leakage into the fiber caused by mechanically induced plasma-membrane breaks that occur in the living animal. Such an artifact is, however, inconsistent with several observations. First, RSA labeling of fibers was reproducibly observed to be concentrated around sites of experimentally induced mechanical stress to fiber membranes, e.g. around needle injection sites. We could therefore directly demonstrate the effectiveness of RSA in specifically labeled mechanically damaged fibers. Second, labeling increased when mechanical stress was applied to fibers by the animal itself, e.g., by downhill running. It would be difficult to explain such an increase as being due to a processing artifact since control (unexercised) and exercised rats were processed identically. Third, the pattern of fiber labeling was invariant from one serial section to another. This result is inconsistent with entry of the albumin during the pickup of the section onto the microscope slide after frozen sectioning, when the cut fibers would be most vulnerable during processing to diffusive movement of any extracellular, incompletely fixed albumin into their cytosol. Such movement would be expected to occur at random across the sectioned muscle since all fibers are exposed by the sectioning to extracellular albumin incompletely washed away by the saline perfusion. Fourth, an alternative label for fiber wounding, FDxLys gave a similar pattern in unexercised muscle, providing an independent confirmation of the occurrence of fiber wounding. Finally, use of RSA as a label for wounded cells has previously been validated in skin.[15]

Exercise-induced Wounding

The most striking finding of our study was the increase in the number of wounded fibers in the tricep (medial head) immediately after eccentric exercise (downhill running). Our interpretation is that these membrane wounds, at least initially, were survived (i.e. resealed) by the muscle

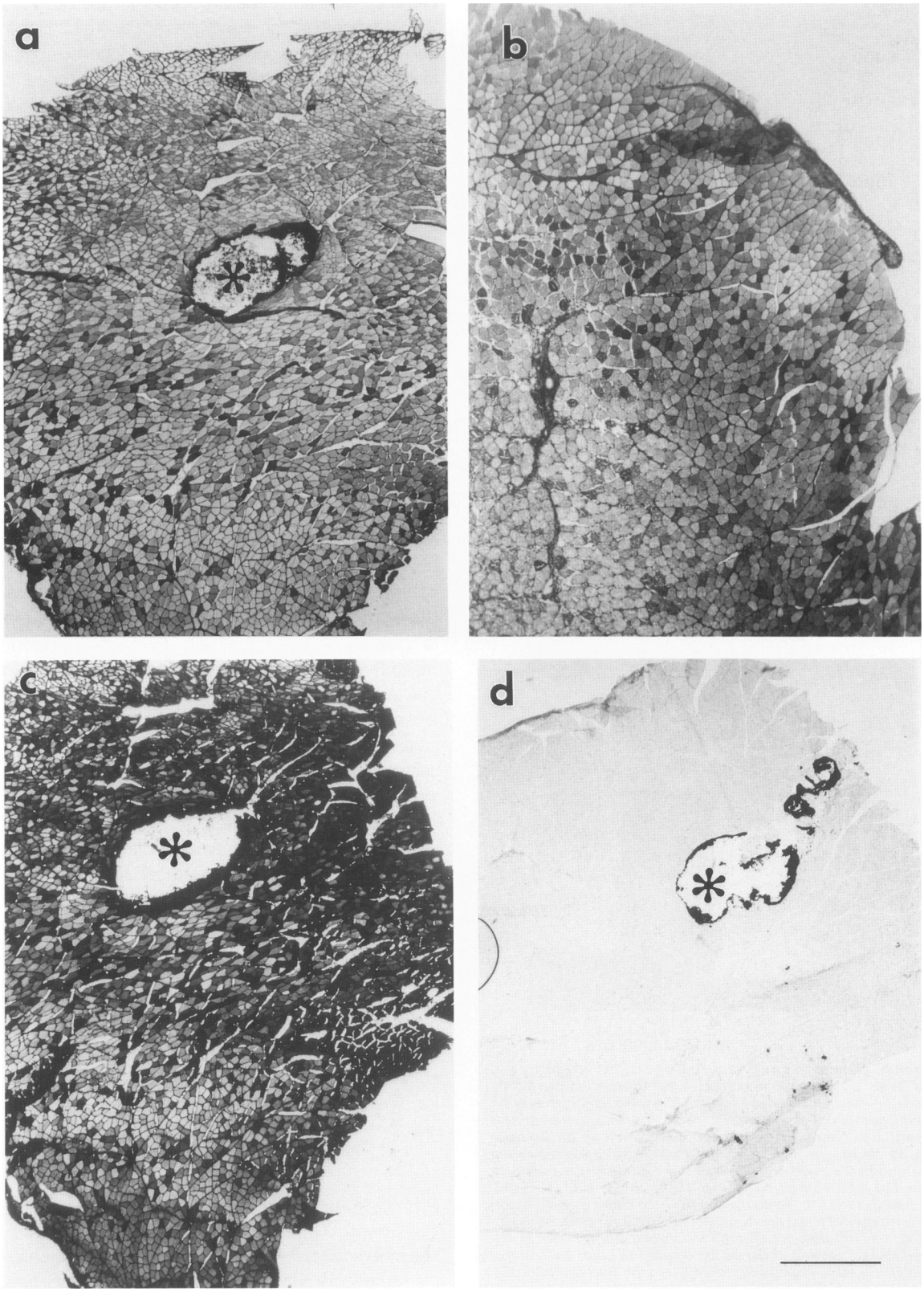

Figure 4. *Photomicrographs of long heads of triceps injected with HRP and obtained* (a) *3 hours after injection and* (b) *24 hours after injection. The section in* (c) *is a serial section of the muscle in* (a) *but was additionally stained with anti-RSA. The muscle shown in* (d) *was injected with PBS and reacted with DAB as for* (a), (b), *and* (c) *as a control to indicate endogenous levels of peroxidase activity. An asterisk (*) marks the injection site that is lined with carbon particles. Bar = 1000 μm.*

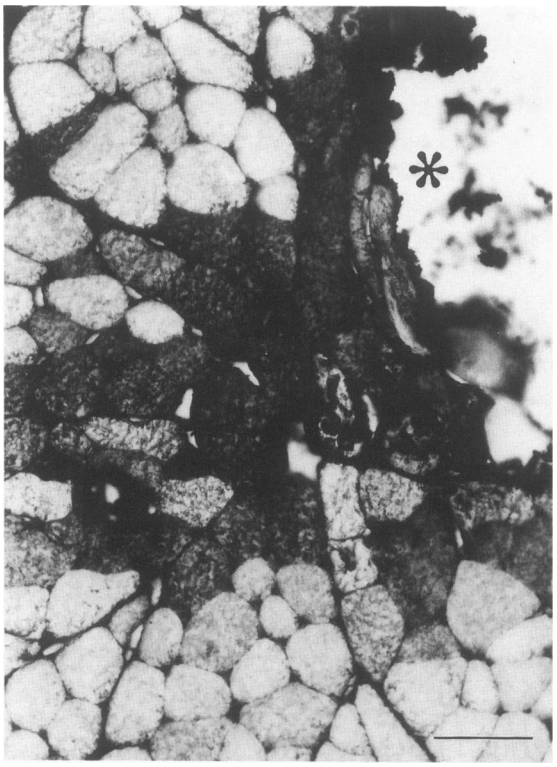

Figure 5. *Photomicrograph of a long head of the triceps injected with PBS from a rat sacrificed 3 hours afterwards, and stained with anti-RSA. An asterisk (*) marks the injection site which is lined with carbon particles. Bar = 200 μm.*

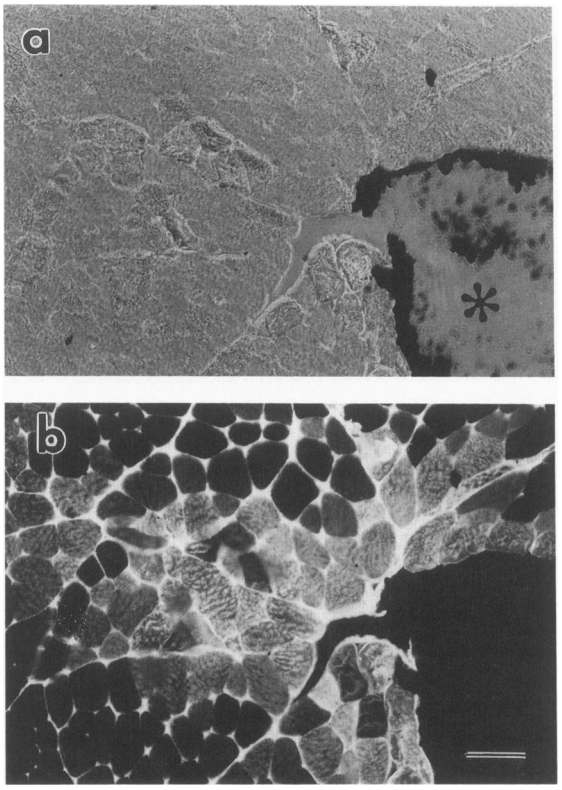

Figure 6. *Phase* **(a)** *and fluorescence* **(b)** *micrographs of a long head injected with PBS only from a rat sacrificed 24 hours afterwards and stained with an anti-RSA antibody coupled to an FITC rather than HRP, as in the above micrographs. An asterisk (*) marks the injection site which is lined with carbon particles. Bar = 200 μm.*

fibers. First, we saw no histologic evidence for frank fiber destruction in frozen or paraffin sections, either immediately after exercise or 24 hours later. Second, labeled cells measurable 24 hours after exercise were fewer in number than immediately after exercise. If each fiber wounded during exercise was unable to seal its wound and died for this or for any other reason, then RSA entry and thus immunochemical staining would have been increased 24 hours later. This was demonstrated by the fact that muscle cells visibly damaged by a needle puncture wound that was made 24 hours before tissue processing were strongly stained with anti-RSA. Resealing of a highly localized, survivable membrane wound would, on the other hand, allow for RSA diffusion from the localized site of entry, along the entire fiber's length, and/or for intracellular degradation of RSA. Albumin has a particularly short measured half-life in mammalian cells of 16 hours.[19] In muscle fibers that survived membrane wounds, both of these processes, label diffusion and degradation, would result in a measured reduction in labeling intensity over time, consistent with the reduced staining we observed over time in the eccentrically exercised medial head. Finally, measured serum CK levels were higher immediately after exercise than 24 hours later, consistent again with an initial, acute injury to the muscle that was not followed in the subsequent 24 hours by fiber death and degradation. A similar CK profile was obtained by Armstrong et al[10] whose experimental conditions for rat exercise were duplicated here.

Our observations provide direct evidence that increased permeability of muscle fiber plasma membranes is an early event in the eccentrically exercised muscle. This change in permeability does not occur uniformly in all fibers but, apparently, at random in the muscle popu-

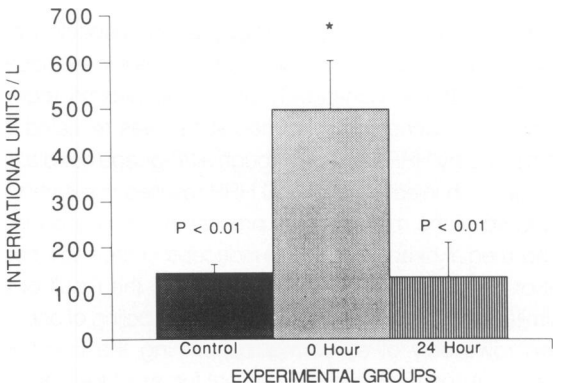

Figure 7. *Serum creatine phosphokinase (CK) levels in control, 0-hour, and 24-hour animals. Error bars represent SEM, and* P *values relative to the 0-hour animal (asterisk) are shown.*

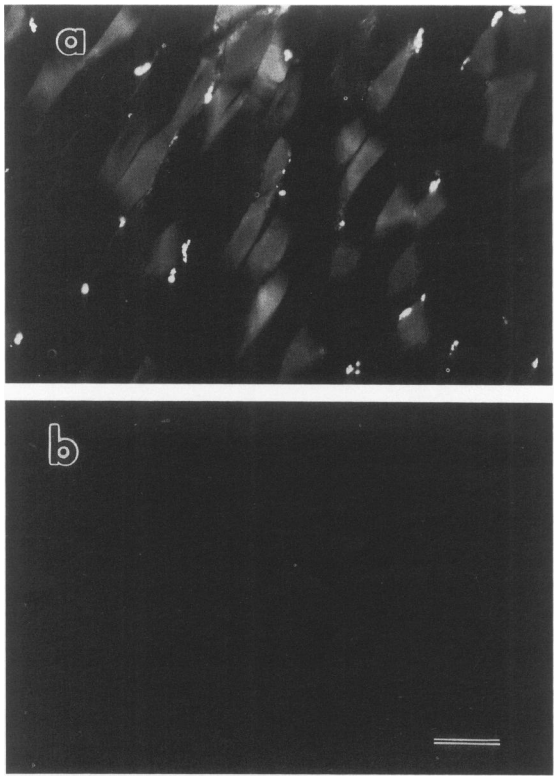

Figure 8. *Fluorescence photomicrographs of long heads taken under identical photographic conditions from:* **(a)** *a rat sacrificed 24 hours after intravenous injection with FDxLys; or* **(b)** *a rat not injected with FDxLys. Bar = 200 μm.*

lation. We suggest that the most likely cause of this change in permeability, which allowed access to the cytosol of a large protein, is a focal plasma membrane disruption caused by the imposition of mechanical force on this fragile structure. Armstrong et al[10] and others[12] have argued that eccentric exercise produces higher forces per cross-sectional area than concentric exercise, stressing thereby, in series, all of the structural elements of the muscles. Both forms of exercise, on the other hand, metabolically stress the fibers. Thus, structural rather than metabolic damage has previously been suspected as the cause of muscle damage during eccentric exercise.

The extreme vulnerability of muscle plasma membranes to mechanically induced stress was revealed in this study by HRP injection through a 26-guage needle. A single such injection of 200 μl HRP resulted in extensive labeling of the muscle fibers present in the triceps long and medial heads and the quadriceps cross-sectioned at or near the injection site. By contrast, the result of a similar injection protocol in skin was the labeling of one or two rows only of epidermal cells lining the injection tract.[15] Apparently, the mechanical forces of the needle puncture and/or the hydrostatic forces generated by the volume of the injection were readily transmitted throughout the entire diameter of the muscle, inducing widespread plasma membrane disruptions. An additional factor contributing to the vulnerability of the muscle to a needle puncture may be the size (especially the great length) of the fibers, and hence their greater likelihood of being directly hit by the needle.

Mechanically generated plasma membrane disruptions, especially if transient and/or highly localized, would not be immediately detectable by conventional histological techniques. Interestingly, however, focal areas of fiber damage were detectable in the electron microscope immediately after eccentric exercise in humans, and one explanation offered for this damage was focal membrane wounding.[8,20] Moreover, 1-μm plastic sections of 0-hour medial heads exercised by downhill running (as in this study) revealed focal disruptions of sarcomeres and a lack of clear delineation between myofibers interpreted as possible sarcolemma disruptions.[10] The eccentrically exercised soleus muscle displayed similar lesions, including disruptions of the A-band and localized dissolution of Z-lines.[12] We propose that initially resealable and/or highly localized but unsealable membrane wounds might, conceivably, initiate damage that progresses along the length of the fiber until, 1–4 days after exercise, it becomes sufficiently severe that it can be readily recognizable by conventional histological techniques. Clearly, macrophages/monocytes infiltrating 1–4 days after exercise[21] could accelerate the destruction of fibers known to occur during this interval.

Further support for this model comes from studies of muscle fibers wounded at their plasma membranes by a microneedle (10–20 μm diameter) puncture.[22] Such fibers initially (< 1 hour after the puncture wound) displayed highly localized regions of damage detectable in the electron microscope that, over the next 2–18 hours, were seen to spread hundreds of microns along the fiber length. By 18 hours after the puncture, surviving "stumps" of the punctured fiber were clearly separated by necrotic segments infiltrated by macrophages and thereby readily identifiable by light microscopic examination. Further progress of the necrosis did not generally occur beyond 18 hours, at which time surviving stumps were seen in the electron microscope to be encased in an intact plasma membrane.

Force produced by the muscle fiber is transmitted across the plasma membrane to the tendon at the myotendinous junction, which has a highly specialized morphology believed to be important in this force transduction role.[23,24] Loading of the myotendinous junction by more than 20% beyond the maximal stress produced by an isometric contraction can, however, lead to its rupture.[25] Although it is not known whether such a structural failure is accompanied locally by a disruption of the plasma membrane, it seems reasonable given its func-

tional role and apparent vulnerability to suspect the myotendinous junction as a possible site of the membrane wounding documented here to occur during eccentric exercise.

Fiber Wounding in Unexercised Rats

We also reproducibly observed muscle fibers that stained with anti-RSA and with an independent tracer, FDxLys, in the triceps (medial head) and several other muscles of the "unexercised," caged laboratory rat. If as argued earlier such labeling is not an artifact of tissue processing, then we can conclude that even a sedentary level of muscular activity is sufficient to wound a small fraction of muscle fiber plasma membranes. Clearly, an important but untested prediction of this hypothesis would be that in the complete absence of muscle activity, fiber wounding would not occur.

In possessing cells wounded at their plasma membranes, normal, undisturbed rat muscle and eccentrically exercised muscle appears to resemble gut and skin.[14,15] In each of these tissues, mechanical forces are normally imposed on constituent cells, and it is these forces, we have suggested, that result in membrane disruptions. We have further proposed that such disruptions provide a route in to and out of cell cytoplasm distinct from the conventional, membrane-bounded routes of endo- and exocytosis.

Technical Applications of Fiber Wounding and Resealing

Wolff et al[26] reported that a simple needle-mediated injection of pure RNA and DNA expression vectors into rat muscle results in the expression of the proteins coded for by these vectors within a certain proportion of the injected muscle's fibers. Clearly, for this to occur, there must exist some mechanism by which injected polynucleotides can enter into fiber cytoplasm. Two possibilities were offered: 1) polynucleotide diffusion into the T tubules is followed by their transfer to the sarcoplasmic reticulum (by an unknown mechanism) where they are released along with Ca^{++} ions into cytoplasm (by another unknown mechanism), or 2) polynucleotides enter " . . . damaged muscle cells, which then recover."

We suggest, based on the present and other recent work in our laboratory, that the second of these mechanisms seems the most likely. We have shown that the plasma membranes of cells of both gut[14] and skin[15] are transiently disrupted by experimentally imposed, or physiologically generated, mechanical forces, rendering cells of these tissues transiently permeable to large, otherwise impermeant extracellular macromolecules. As mentioned earlier, lining needle puncture wounds in skin are numerous epidermal cells that were transiently permeabilized.[15] Most importantly, we here show using HRP and FDxLys as a tracer for wounding and injection conditions similar to those of Wolff et al[26] that strikingly high levels of muscle cell wounding follows a needle puncture of muscle and fluid injection into the muscle body. Mechanical wounding of cultured cells by scrape loading[27] results, if performed in the presence of DNA expression vectors, in their transfection.[28] In fact, we previously proposed that the occurrence of cell plasma membrane wounding and resealing *in vivo* suggested methods " . . . for selectively loading into cells of tissues and organs such experimentally useful molecules as . . . deoxyribonucleic acid . . . ".[14]

It seems reasonable to suggest therefore that the needle puncture of muscle used by Wolff et al[26] transiently disrupted fiber plasma membranes, allowing direct entry into wounded fiber cytoplasm of the RNA and DNA vectors. Alternatively, the fibers of muscle might have been wounded subsequent to the injection by mechanical forces imposed on them during normal animal (rat) locomotion, as shown here. Thus we would suggest that gene transfer *in vivo* by the new method of Wolff et al[26] may be considerably improved by repeatedly penetrating the muscle with the needle while continuously injecting the DNA, and/or by inducing the injected animal to exercise on a treadmill after injection. Moreover, as our results indicate, genes may be transferred into the cells of other tissues by an injection, and/or by the additional application after the injection of other forms of mechanical force capable of wounding cell membranes *in vivo*.

Role of Fiber Wounding in Growth Control

Membrane wounding in muscle may directly initiate not only damage but also the cellular processes, such as chemotaxis and proliferation, necessary for its repair. Such cell-mediated processes are generally believed to be mediated by polypeptide growth factors. Basic fibroblast growth factor (bFGF) is one of several growth factors now suspect as being biologically active in muscle growth control, and we here will suggest that its release in muscle could be directly coupled to plasma membrane wounding of muscle fibers.

Muscle contains bFGF,[29,30] and cultured muscle fibers respond to this polypeptide with increased growth.[31] In fact, exercise apparently increases muscle expression of bFGF.[32] Finally, higher than normal levels of bFGF have been observed to be present in the extracellular matrix of dystrophic mouse muscle,[33] which like exercise-damaged muscle contains dead and degenerating fibers.[34]

We have shown elsewhere that bFGF, which lacks the classical signal peptide sequence for export by exocytosis, is released through cell membrane disruptions,[35,36] such as those shown here to occur in eccentrically exercised and even in unexercised muscle. Therefore, bFGF might be released through muscle fiber plasma membrane breaks. This mechanism could initiate growth in muscle damaged by exercise, in muscle undergoing hypertrophy in response to exercise, and/or in undamaged muscle in which maintenance of normal mass depends on muscle usage. Our work suggests that myofiber plasma membrane wounding occurs under each of these conditions.

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