Peroxynitrite induces contractile dysfunction and lipid peroxidation in the diaphragm


Supinski, G., D. Stofan, L. A. Callahan, D. Nethery, T. M. Nosek, and A. DiMarco. Peroxynitrite induces contractile dysfunction and lipid peroxidation in the diaphragm. J. Appl. Physiol. 87(2): 783–791, 1999.—Peroxynitrite may be generated in and around muscles in several pathophysiological conditions (e.g., sepsis) and may induce muscle dysfunction in these disease states. The effect of peroxynitrite on muscle force generation has not been directly assessed. The purpose of the present study was to assess the effects of peroxynitrite administration on diaphragmatic force-generating capacity in 1) intact diaphragm muscle fiber bundles (to model the effects produced by exposure of muscles to extracellular peroxynitrite) and 2) single skinned diaphragm muscle fibers (to model the effects of intracellular peroxynitrite on contractile protein function) by examining the effects of both peroxynitrite and a peroxynitrite-generating solution, 3-morpholinosydnonimine, on force vs. pCa characteristics. In intact diaphragm preparations, peroxynitrite reduced diaphragm force generation and increased muscle levels of 4-hydroxynonenal (an index of lipid peroxidation). In skinned fibers, both peroxynitrite and 3-morpholinosydnonimine reduced maximum calcium-activated force. These data indicate that peroxynitrite is capable of producing significant diaphragmatic contractile dysfunction. We speculate that peroxynitrite-mediated alterations may be responsible for much of the muscle dysfunction seen in pathophysiological conditions such as sepsis.

free radicals; skeletal muscle; respiratory muscles

METHODS

Animal usage. Animals were cared for in the Case Western Reserve University animal facilities in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Animals were provided food and water ad libitum before the experiments.

All studies were performed in diaphragm muscle samples taken from adult rats (Zivic-Miller, 400–600 g). When diaphragms were harvested, rats were first anesthetized in a halothane chamber and then decapitated. For intact muscle fiber bundle studies, one portion of the diaphragm was placed in a dissecting dish, muscle strips were cut from this muscle, and strips were mounted in an organ bath. Details of this procedure are provided in intact muscle fiber bundle studies: determination of force generation. The remaining diaphragmatic muscle was frozen in liquid nitrogen, stored, and later used for assessment of lipid peroxidation.

For skinned fiber studies, animals were also killed by decapitation, and the diaphragm harvested. After the diaphragm was removed, this muscle was rinsed in physiological saline and cut into pieces ~5 mm wide. These diaphragm sections were stored in 50% CTP relaxing 50% glycerol solution at ~20°C until the time of study. Fibers were used within 1 wk for skinned muscle fiber studies, although muscles stored in this fashion remain usable for at least 3 mo. Details regarding force assessment in skinned fibers are provided in Skinned muscle fiber studies: SIN-1 protocol.
Intact muscle fiber bundles: determination of force generation. After animal death, diaphragm sections were placed in a dissection dish containing Krebs-Henseleit solution bubbled with 95% O2-5% CO2, with a pH of 7.4 at 27°C. Paired muscle strips were then dissected from the diaphragm; during this dissection, care was taken to include both a portion of the central tendon and the rib insertion site of each strip. Paired strips were then mounted vertically in paired, water-jacketed organ baths (Radnoti Glass, Monrovia, CA) maintained at 27°C and containing Krebs-Henseleit solution bubbled with 95% O2-5% CO2 (24). The rib end of each strip was secured to the bottom of its organ bath by a silk tie, and the central tendinous end of each strip was attached to a Grass FT10 force transducer (Grass Instruments, Quincy, MA) by means of a steel rod. Platinum field electrodes were placed around each muscle strip; electrodes were connected to a constant-current amplifier (Biomedical Technology of America, Cleveland, OH) that was attached, in turn, to a Grass S488 stimulator. The length of each muscle strip was subsequently adjusted to optimum length (i.e., the length at which force generation is maximal), and the current was adjusted to a supramaximal level (i.e., 120% of the current required to achieve a maximal twitch force). Diaphragm strips were next stimulated sequentially with trains of 1-, 20-, and 50-Hz electrical stimulation to provide a baseline assessment of each muscle's force-frequency relationship.

An aliquot of concentrated solution of peroxynitrite (100 µl peroxynitrite in 6 mM NaOH and 6 mM NaCl; Upstate Biotechnology, Lake Placid, NY) was then added to one of the organ baths, whereas an equivalent volume of diluent (i.e., 100 µl of 6 mM NaOH and 6 mM NaCl) was added to the second bath. Serial matched assessments of the diaphragm force-frequency relationship (i.e., sequential stimulation at 1, 20, and 50 Hz) were performed at 30-min intervals for both muscle strips. At 120 min after the addition of peroxynitrite or diluent to organ baths, muscles were removed, rapidly frozen in liquid nitrogen, and stored for later biochemical analysis.

Intact muscle fiber bundle studies: measurement of TBAR and 4-HNE. We measured thiobarbituric acid-reactive substances (TBAR) and 4-hydroxynonenal (4-HNE) levels, two indicators of lipid peroxidation, in three muscle samples from each animal used for intact muscle fiber bundle studies (8, 19). Specifically, these levels were assessed in a sample of diaphragm obtained immediately after animal death and in the two muscle strips used for physiological assessment of muscle function (i.e., one incubated in peroxynitrite and the second incubated in diluent for 120 min). For TBAR measurements, a modification of the technique of Janero (15) was used. Muscle samples of ~40 mg were pulverized under liquid nitrogen, added to 20 mM Tris·HCl (pH 7.4) at a 10% dilution, and then homogenized at 4°C for 2 min. This homogenate was then centrifuged at 3,000 g for 10 min at 4°C; the resulting supernatant was collected and stored on ice. For the measurement of TBAR, 200 µl of supernatant were added to 650 µl of 3.4 mM N-methyl-2-phenylinodole in acetonitrile and vortexed for 3–4 s. We then added 150 µl of 37% HCl and incubated this reaction mixture at 45°C for 60 min. The reaction mixture was then cooled on ice, and its absorbance was read spectrophotometrically (model UV 1201, Schimadzu) at 586 nm. Standard curves were constructed by using reference samples of malondialdehyde, and concentrations in experimental samples were calculated by referring to the malondialdehyde standard curve.

For measurements of 4-HNE (8), 200 µl of each initial homogenate were added to 650 µl of N-methyl-2-phenylinodole and vortexed. Afterward, 150 µl of 15.5 µM methanesulfonic acid were added, and the homogenate was incubated at 45°C for 40 min. The mixture was then cooled on ice, and its absorbance was read at 586 nm. The concentration of 4-HNE per gram wet weight of tissue was calculated by reference to standard curves generated using known concentrations of pure 4-HNE.

Skinned muscle fiber studies: solutions. The composition of solutions used for skinned muscle fiber studies was calculated by using a computer program (Borland International, Scotts Valley, CA) that takes into account stability constants and stock solutions to produce final solutions of the correct ionic strength and pCa (12). The skinning solution used was composed of (in mM) 50 potassium methane sulfonate, 15 phosphocreatine, 10 EGTA, 1 Mg2+, 2 MgATP, and 20 imidazole, with ionic strength 150, pCa >8.5, and pH 7.0 at 22°C (18). A CTP relaxing solution consisting of (in mM) 110 potassium methane sulfonate, 5 EGTA, 1 Mg2+, 2 MgCTP, and 20 imidazole, with ionic strength 150, pCa >8.5, and pH 7.0, was employed for diaphragm muscle storage. To prevent phosphorylation of the myosin light chains in these stored muscles, CTP was used in place of ATP, because CTP is not a substrate for myosin light chain kinase. All solutions contained protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 10 µM aprotinin, and 1.0 mM benzamidine) to prevent muscle breakdown during experimental manipulation.

Skinned muscle fiber studies: fiber mounting. For preparation of skinned muscle fibers, we first placed a diaphragm section in skinning solution and allowed this muscle to warm to room temperature. With the use of a dissecting microscope, small bundles of ~10 fibers were pulled away from the whole section. These bundles were incubated in 0.1% Triton X-100, anionic detergent that permeabilizes all membranes, for 30 min on ice. The bundles were placed back in the skinning solution, and individual diaphragm fibers were separated from these bundles. Individual fibers were then mounted between two platinum posts. One of these posts was fixed to an overhead support, and the other was connected to a micro force transducer (Harvard Apparatus, South Natick, MA). The transducer was, in turn, attached to a movable gear assembly (thereby allowing fiber length to be adjusted). Muscle fiber sarcomere length was adjusted to 2.6 µm by using helium-neon laser diffraction. Fibers were initially immersed in a solution of pCa 8.5 (i.e., a solution containing essentially no calcium) (18).

Skinned muscle fiber studies: SIN-1 protocol. Two groups of diaphragm fibers were studied in this protocol: 1) control, non-SIN-1-exposed fibers (n = 4), and 2) fibers exposed to SIN-1 (Calbiochem, La Jolla, CA), a compound that spontaneously decomposes in aqueous solutions to yield superoxide and nitric oxide, thereby forming peroxynitrite (n = 4) (7). A baseline force vs. pCa curve was constructed for each fiber by sequential immersion in solutions of increasing calcium concentration (i.e., pCa 6.00, 5.90, 5.80, 5.75, 5.70, 5.65, 5.60, 5.55, 5.50, 5.40, 5.30, 5.20, and 5.00). Calcium-activated force was recorded on a Gould 2400 chart recorder (Gould, Cleveland, OH). Control fibers were then incubated in a no-calcium (pCa 8.5) relaxing solution for 5 min. SIN-1 group fibers were incubated in a pCa 8.5 solution to which 500 µM SIN-1 had been added. This particular concentration of SIN-1 decomposes to yield 5 µM peroxynitrite/min (assessed by using a spectrophotometric assay; Ref. 1). At the end of the 5-min incubation, the force vs. pCa curve was repeated. The
diameter of each fiber was then measured by using a micrometer mounted in one of the eyepieces of the microscope.

Skinned muscle fiber studies: peroxynitrite protocol. The experimental details of this protocol were essentially identical to those in the SIN-1 protocol, except for the agents in which the skinned fibers were incubated. In this protocol, skinned diaphragm muscle fibers were incubated for 5 min in either 1) pCa 8.5 solution to which 1 mM peroxynitrite (final concentration; this was added as a 40-µl bolus in diluent containing 6 mM NaOH and 6 mM NaCl) had been added, or 2) pCa 8.5 solution ("relaxing solution," 2 ml) containing 40 µl of the diluent vehicle in which the peroxynitrite was dissolved (the diluent contained 6 mM NaOH and 6 mM NaCl; this addition had almost no effect on solution pH, raising it to only 7.02 from 7.00). The protocol in brief, therefore, was 1) control force vs. pCa curve construction, 2) 5-min fiber incubation, 3) repeat of force vs. pCa curve after exposure, and 4) measurement of muscle fiber diameter.

Data analysis. For intact muscle fiber studies, muscle force was normalized for cross-sectional area (CSA), which was calculated as CSA = muscle weight/1.06 × muscle length. For intact fiber studies, diaphragm contraction time was defined as the time required for peak force development during muscle stimulation with a single supramaximal impulse. Half relaxation time was defined as the time required for force to fall by 50% from its peak value.

For skinned muscle fibers, force was also normalized for CSA. For these latter studies, fibers were assumed to be cylindrical, and the diameter of each fiber was measured directly during data collection. CSA was calculated as π(D²)/4, where D is diameter. Forces were normalized for CSA and expressed in kilopascals.

With the use of computer software (SigmaPlot, SPSS), the force vs. pCa relationship for skinned fibers was fit to the Hill equation

\[ F_{\text{max}} = 100\left[\frac{[Ca^{2+}]}{IC_{50}}\right]^n + \left(Ca_{90}\right)^n \]

where \( F_{\text{max}} \) is maximum calcium-activated force, \( Ca_{90} \) is the Ca²⁺ concentration ([Ca²⁺]) producing half-maximal activation, and \( n \) is the Hill coefficient (13). The \( Ca_{90} \) was used as an index of calcium sensitivity, and the Hill coefficient was used as a measure of the steepness of the curve.

All data are expressed as means ± 1 SE.

RESULTS

Intact muscle fiber bundles: muscle strip characteristics. As expected, intact muscle fiber bundles incubated in diluent and peroxynitrite had similar muscle lengths (optimum length) (2.3 ± 0.2 and 2.3 ± 0.1 cm, respectively) and weights (49 ± 10 and 44 ± 11 mg, respectively). Maximal baseline (i.e., pre-peroxynitrite or diluent administration) force generation in response to 50-Hz stimulation (\( F_{\text{max}} \)) for diluent and peroxynitrite-treated muscle strips was also similar, averaging 28 ± 1 and 29 ± 1 N/cm², respectively.

Intact fiber bundles: effect of peroxynitrite on muscle force. Peroxynitrite administration did not alter contraction time (Fig. 1, top), but did elicit a progressive increase in half relaxation time, as shown in Fig. 1, bottom. Specifically, baseline half relaxation time (i.e., before peroxynitrite application) was 63 ± 5 and 62 ± 2 ms, respectively, for peroxynitrite and diluent experimental groups; by the conclusion of the 120-min incubation period, half relaxation time increased to 87 ± 3 ms in the peroxynitrite-treated strips (\( P < 0.01 \)) but remained at 67 ± 4 ms (i.e., close to baseline levels) in diluent-treated muscles. Peroxynitrite incubation also produced significant reductions in muscle force generation in response to 1-, 20-, and 50-Hz activation, as shown in Fig. 2. Over the 120-min incubation period, 1-Hz force fell by 29 ± 2 and 63 ± 3% in diluent-treated control and peroxynitrite-treated groups (\( P < 0.01 \)), whereas 20-Hz force fell 19 ± 1 and 52 ± 2%, respectively, in these groups (\( P < 0.01 \)), and 50-Hz force fell by 11 ± 1 and 38 ± 2%, respectively (\( P < 0.01 \)).

Intact fiber bundles: effect of peroxynitrite on lipid peroxidation. Peroxynitrite elicited a significant increase in diaphragm muscle 4-HNE levels, compared with time-matched, diluent-treated control muscles, as shown in Fig. 3, top (\( P < 0.05 \)). 4-HNE levels for muscles incubated in diluent alone for 120 min were equal to 4-HNE levels measured in diaphragm muscle samples freshly frozen immediately after animal death.

There was no difference in TBAR formation in peroxynitrite-treated muscles compared with diluent-treated controls (Fig. 3, bottom). Unlike 4-HNE levels, TBAR levels for muscles incubated for 120 min in diluent were significantly higher (\( P < 0.05 \)) than levels measured in
muscles frozen at the time of death, indicating a significant degree of nonspecific formation of TBAR over time in incubated control muscles.

Skinned fiber force generation: SIN-1 protocol. The baseline force vs. pCa curves from control and SIN-1 fiber groups were very similar. For example, $F_{\text{max}}$ was $126 \pm 4$ kPa for control fibers and $128 \pm 5$ kPa for SIN-1 fibers. There were also no differences in $C_{\text{a50}}$ or in the Hill coefficient between the two groups. Incubation of skinned fibers in SIN-1, however, resulted in reductions in $F_{\text{max}}$. These data are plotted in Fig. 4. The maximum force of fibers exposed to SIN-1 for 5 min was $107 \pm 5$ kPa, a decrease of 17% of its baseline value ($P < 0.02$). The maximum force of control fibers fell only slightly, to $118 \pm 4$ kPa, after incubation in pCa 8.5 ($P < 0.02$ for comparison of postincubation $F_{\text{max}}$).

In addition to reducing $F_{\text{max}}$ production, SIN-1 exposure produced a change in the $-\log C_{\text{a50}}$ ($pC_{\text{a50}}$) in single diaphragm fibers. The $pC_{\text{a50}}$ dropped from a preexposure value of $5.84 \pm 0.02$ to $5.76 \pm 0.02$ in fibers incubated in SIN-1 ($P < 0.005$); there was no statistically significant difference in the control group (i.e., $5.80 \pm 0.03$ to $5.75 \pm 0.03$). This difference is represented in Fig. 5, in which calcium-activated forces are normalized to maximum tension. The center of the postincubation SIN-1 curve is shifted to the right, indicating a decrease in the calcium sensitivity of the contractile proteins after exposure to SIN-1. SIN-1 incubation did not produce any change in the Hill coefficient, which averaged $4.14 \pm 0.33$ before SIN-1 and $3.44 \pm 0.31$ after SIN-1 exposure.

Skinned fiber force generation: peroxynitrite protocol. As in the SIN-1 protocol, there were no differences observed between the baseline force vs. pCa curves of the control group and those of the peroxynitrite group; $F_{\text{max}}$ was $112.2 \pm 2.3$ and $110.0 \pm 5.2$ kPa, respectively. When control group diaphragm fibers were incubated...

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**Fig. 2.** Effect of peroxynitrite on forces generated by intact diaphragm fiber bundles. Displayed forces generated in response to single stimuli (i.e., twitch force; top), in response to 20-Hz stimulation (middle), and in response to 50-Hz stimulation (bottom) are shown. Symbols are as defined in Fig. 1 legend. Error bars indicate ±1 SE. *$P < 0.01$, peroxynitrite-treated muscles vs. control.

**Fig. 3.** 4-Hydroxynonenal (top) and thiobarbituric acid-reactive substances (bottom) levels measured in samples taken from intact diaphragm fiber bundles. Shown are data for samples taken from intact diaphragm fiber bundles. Shown are data for samples taken from diaphragm at time of animal death, control muscles incubated in Krebs-Henseleit solution, and muscle bundles incubated in Krebs-Henseleit solution containing peroxynitrite. Error bars indicate ±1 SE. *$P < 0.05$, peroxynitrite vs. control.
for 5 min in relaxing solution (2 ml) containing an aliquot of the diluent used for the peroxynitrite (i.e., 40 µl of 6 mM NaOH and 6 mM NaCl), then reactivated with calcium solutions, the resulting force vs. pCa curves were essentially identical to baseline curves. Diaphragm skinned fibers responded quite differently to incubation in 1 mM peroxynitrite (i.e., 2 ml of relaxing solution containing a 40-µl bolus of peroxynitrite to provide a final concentration of 1 mM peroxynitrite), with Fmax of this fiber group decreasing by 18% from baseline (P < 0.02) to 90.1 ± 2.8 kPa (P < 0.02 for comparison of postincubation control Fmax to postincubation peroxynitrite Fmax). These curves are displayed in Fig. 6.

The pCa50 of muscle fibers was not altered by peroxynitrite, with values for this parameter of 5.87 ± 0.02 before incubation and 5.82 ± 0.01 after peroxynitrite incubation. The Hill coefficients were unchanged by peroxynitrite, averaging 4.22 ± 0.46 for control fibers before incubation, 4.32 ± 0.64 for control fibers after incubation, 4.71 ± 0.50 for peroxynitrite group fibers before incubation, and 4.50 ± 0.68 for peroxynitrite group muscle fibers after peroxynitrite exposure.

**DISCUSSION**

These data indicate that peroxynitrite administration induces 1) significant reductions in diaphragm force generation in intact muscle fibers, 2) alterations in twitch kinetics in intact fibers (i.e., producing a slowing of muscle relaxation), 3) lipid peroxidation in intact fibers, and 4) reductions in the Fmax of the contractile protein force vs. pCa relationship of skinned muscle fibers.

Peroxynitrite and SIN-1 dosages used in the present study. When interpreting the results of this study, it is important to consider what might be the expected rates of generation of peroxynitrite under in vivo conditions. Unfortunately, no measure has yet been made of peroxynitrite generation rates in muscle, although an “upper” estimate of this parameter can be made based on reference to recent measurements of nitric oxide and...
superoxide generation by mitochondria (11). Superoxide is generated at a rate of 1.2 nmol·min\(^{-1}\)·mg protein\(^{-1}\), and nitric oxide is generated at rates of 1.4–7.1 nmol·min\(^{-1}\)·mg protein\(^{-1}\) by normal mitochondria (11); if the two species react quantitatively with each other, a peroxynitrite generation of 1.2 nmol·min\(^{-1}\)·mg would be expected. Assuming a muscle density of 1.06, this would translate into a theoretical maximum generation rate of 1 mM/min by mitochondria. In real physiological situations, however, much lower rates (0.1–1.0 µM/min) are generally thought to occur. Based on this analysis, it is conceivable that 0.1–1.0 µM/min peroxynitrite generation rates may exist in muscles under appropriate conditions.

When comparing the doses of peroxynitrite and SIN-1 tested in the present experiment with the "usual" rates at which peroxynitrite is thought to be generated in vivo, it is important to recognize that peroxynitrite "dosage" should be calculated as a concentration × time product (see Refs. 3 and 9 for formulas). According to this approach, the peroxynitrite concentration × time product produced by our peroxynitrite "bolus" administration would be 26 µM/min, whereas the concentration × time product resulting from our SIN-1 administration would be 25 µM/min. These concentration × time products would be similar to those seen by exposure of muscles to a 0.125 µM/min generation rate (a rate well within the physiological realm) for 200 min. The fact that the concentration × time products for the paradigms of peroxynitrite and SIN-1 exposure used in the present study are virtually the same probably accounts for the fact that these two agents produced comparable reductions in the \(F_{\text{max}}\) of single muscle fiber force vs. pCa curves.

We should also note that, in the studies in which we examined the effects of exogenously administered peroxynitrite in intact muscle fiber bundles, it is likely that these muscle were endogenously generating reactive oxygen species at a low rate, as previously demonstrated (22). This "mimics" the situation in "real" pathophysiological situations (e.g., ischemia-reperfusion) in which muscle fibers may be exposed to radical species, including peroxynitrite, generated exogenously by white blood cells and endothelial cells. The difference between the responses, over time, of control and peroxynitrite-treated groups (in Figs. 1–3) reflects, presumably, the direct and indirect effects of the addition of exogenous peroxynitrite to this situation.

Relationship to previous studies. Recent reports have suggested that oxygen-derived free radicals play an important role in the development of skeletal muscle dysfunction in a number of physiological and pathophysiological situations. As an example, both Shindoh et al. (25) and Surell et al. (31) have shown that administration of free radical scavengers reduces the magnitude of the decrement in muscle force-generating capacity observed during the development of endotoxin-mediated sepsis in rodents. In other work, several investigators have shown that markers of free radical-mediated lipid peroxidation increase in fatiguing muscle (21, 30). Moreover, this work has also shown that administration of free radical scavengers both blunts the increase in these markers and slows the rate of development of muscle fatigue (30).

These previous studies, however, have not completely defined either the specific mechanisms of production or the specific molecular species of free radical responsible for damaging muscle in sepsis and fatigue. One possibility is that the hydroxyl anion is the principal molecular species responsible for contributing to the development of muscle dysfunction in these conditions. This argument is supported by the fact that both contraction and sepsis-induced muscle dysfunction can be reduced by the administration of DMSO, a commonly used hydroxyl radical scavenger (26, 30). The fact that muscle dysfunction in these two situations is also decreased by administration of superoxide scavengers (i.e., superoxide dismutase) and agents that degrade hydrogen peroxide (i.e., catalase) could be due to the fact that both superoxide and hydrogen peroxide are substrate for hydroxyl ion generation by the iron-catalyzed Haber-Weiss reaction (26, 30).
Another possibility is that the peroxynitrite ion (the reaction product of superoxide and nitric oxide) is the major cause of free radical-related muscle damage in sepsis and fatigue. This latter argument is supported by the fact that markers of peroxynitrite-mediated protein modification (i.e., nitrotyrosine formation) are easily detected in muscle after the induction of sepsis (29). In addition, administration of NOS inhibitors almost entirely prevents the development of sepsis-related muscle dysfunction and reduces nitrotyrosine formation in muscle (4). Both of these findings would be difficult to explain if one assumes that the hydroxyl radical alone is responsible for muscle dysfunction in sepsis. Both findings are, however, consistent with a role for peroxynitrite in mediating sepsis-related muscle dysfunction, because peroxynitrite is the reaction product of nitric oxide with superoxide. In addition, whereas an argument could be made that the effect of NOS inhibitors to prevent muscle dysfunction in sepsis may simply result from an inhibition of production of nitric oxide per se, nitric oxide alone does not elicit formation of nitrotyrosine, and nitric oxide per se has not been reported to produce alterations in muscle contractile function that are of sufficient magnitude to account for the changes in force-generating capacity observed in muscle during the development of sepsis (21). Taken together, these considerations argue that the peroxynitrite anion may play a role in the development of sepsis-induced muscle dysfunction.

If a given free radical species does, in fact, contribute to the development of muscle dysfunction in sepsis and other pathophysiological conditions, then not only should inhibitors of the production of that species prevent the development of muscle dysfunction, but also direct administration or generation of that species in tissue should induce a form of dysfunction akin to that observed in the pathophysiological condition being studied. For this reason, several investigators have examined the effects produced in muscle and muscle constituents by superoxide anions, nitric oxide, hydrogen peroxide, and hydroxyl radicals (5, 6). Callahan et al. (6) found that direct exposure of Triton-skinned single diaphragm fibers to superoxide or to hydroxyl radicals resulted in a substantial decrease in the maximum force fibers were able to generate (i.e., a reduction in the Fmax of the force vs. pCa relationship). In another study, Broto and Nosek (5) showed that exposing limb single muscle fibers to hydrogen peroxide led to reductions in calcium release from the sarcoplasmic reticulum in response to t-tubular depolarization and ablated sarcoplasmic reticulum calcium-induced calcium release. In addition, Reid (21) provided evidence that nitric oxide affects the shape of the muscle force-frequency relationship, with this curve shifting to the right in the presence of nitric oxide and undergoing a leftward shift under conditions of nitric oxide depletion. Recently, Perkins et al. (20) demonstrated a pronounced effect of nitric oxide to depress the Fmax of Triton-permeabilized single muscle fibers. In this latter work, the effects of nitric oxide were found to be related to an action to directly depress myosin ATPase activity.

Investigations of similar strategy have not, until the present study, been undertaken by using peroxynitrite. The pattern of physiological dysfunction produced by incubation of peroxynitrite with whole muscle cells in the present work (reductions in force-generating capacity, slowing of twitch relaxation, development of lipid peroxidation) has been reported to occur in the diaphragm both during the development of muscle fatigue and in various models of systemic sepsis (25, 26, 28, 30). In addition, the alterations in contractile protein function in skinned fibers (i.e., changes in the force vs. pCa relationship for skinned muscle fibers) induced by exposure to either peroxynitrite per se or a peroxynitrite-generating solution in the present study are similar to those recently described for skinned respiratory and limb muscle fibers taken from muscles of animals made septic by endotoxin administration (27). Inadequate studies of skinned muscle fiber function in fatigued muscle have been done to see whether similar shifts can sometimes be observed after periods of strenuous exercise. Nevertheless, it is apparent that many of the pathophysiological derangements seen in muscle after the development of fatigue or sepsis are reproduced by exposure of intact and skinned fibers to peroxynitrite.

Potential sites of dysfunction. We should note that the two types of peroxynitrite exposure studied in the present study (i.e., incubation of whole muscle strips with this agent and addition of peroxynitrite or a peroxynitrite-generating solution to the medium bathing skinned fibers) are quite different and would be expected to affect different muscle sites. Peroxynitrite is highly reactive, and reaction with cellular constituents should be complete within seconds of exposure. In fact, the half-life of this molecule on exposure to the types of physiological medium used to incubate preparations in the present study (i.e., solutions adjusted to a “normal” physiological pH) has also been estimated to be on the order of 1–2 s (3). As a result, it is likely that peroxynitrite added to intact muscle fibers in the present study reacted with outer cell structures, and it is unlikely that peroxynitrite per se penetrated cells to alter intracellular constituents (e.g., mitochondria) when administered in this fashion. As a corollary, the physiological consequences of exposure to peroxynitrite would be expected to be the direct result of alterations in muscle cell surface receptors and membranes. Effects at these sites could, theoretically, alter action potential propagation and excitation-contraction coupling, thereby directly affecting muscle force generation and relaxation characteristics. In addition, peroxynitrite-mediated damage to outer cell membranes may lead to gradual ionic shifts (i.e., leakage of calcium and sodium into cells and potassium out of cells). By this latter mechanism, peroxynitrite might induce gradually progressing alterations in intracellular metabolism that could, in turn, change sarcoplasmic reticulum and contractile protein function. The potential involvement of some process akin to this latter mechanism is supported by the fact that changes in muscle function seen in intact fiber bundles in the present study appeared to evolve long after the initial exposure to this
substance ended (see Figs. 1 and 2; force changes were not complete until 30–60 min after peroxynitrite exposure). Alternatively, the gradual progression of muscle dysfunction may have reflected a slowly propagating lipid peroxidation chain reaction.

In our skinned fiber studies, the contractile apparatus was directly exposed to peroxynitrite or a peroxynitrite-generating solution, and it seems likely that the alterations in function observed (i.e., reductions in F\text{max} and muscle pCa\text{50} without alterations in the Hill coefficient) represent a direct chemical modification of one or more contractile proteins. In preliminary experiments, we measured force vs. pCa relationships for skinned fibers at different points in time after exposure to peroxynitrite-generating solution to determine the "optimal" time for observing changes. It is worth noting that we found that the shifts we describe in skinned fiber function occurred almost immediately after exposure (within 5 min) and that the magnitude of induced changes in F\text{max} neither increased nor decreased over the subsequent 15-min period after this initial exposure to peroxynitrite. This observation supports the concept that peroxynitrite exposure elicits an immediate and irreversible (or only slowly reversible) alteration in contractile protein function. Determination of the specific molecular alteration induced by peroxynitrite was beyond the intended scope of this study, but it is of interest that the characteristics of the force vs. pCa curve shift induced by peroxynitrite are similar to those previously reported after exposure of skinned fibers to phosphate (phosphate also decreases F\text{max} and pCa\text{50}) (18). It is thought that phosphate elicits this physiological change by altering the interaction between troponin and tropomyosin, thereby reducing access of actin to myosin cross bridges. It is conceivable that peroxynitrite could chemically modify this same site and thereby produce similar, albeit less reversible, alterations in contractile protein function. Phosphate, however, alters the Hill coefficient, and no such alteration was observed in the present study. Another possibility is that peroxynitrite directly altered myosin ATPase, an action that would not affect the Hill coefficient but would reduce F\text{max} in the fashion observed.

Potential sources of peroxynitrite generation in intact muscles. The observations made in the present report are only relevant to pathophysiological conditions in which muscle fibers are exposed to peroxynitrite in a manner similar to that examined in this study. Our exposure of intact fiber bundles to peroxynitrite should model conditions under which peroxynitrite is generated at the surface of muscle cells. Several reports have indicated that superoxide anions are released from contracting muscle fibers (22); under such conditions, superoxide may well react with nitric oxide generated by either endothelial cells or myocytes themselves to form peroxynitrite in the extracellular space adjacent to the muscle fiber surface. Infiltration of neutrophils into muscle, a phenomenon observed after ischemia-reperfusion, sepsis, and eccentric contraction-induced muscle injury, may also result in a situation in which large quantities of superoxide and peroxynitrite are formed in close proximity to the muscle cell surface (10, 23).

Peroxynitrite is also generated within cells and may reach especially high concentrations in mitochondria as both of the substrates (superoxide and nitric oxide) required for formation of peroxynitrite are generated in relatively high quantities in this organelle (superoxide is a normal by-product of mitochondrial respiration, and high concentrations of NOS are localized to mitochondria) (21). Peroxynitrite formed in or near mitochondria may be available for reaction with a variety of intracellular constituents and may well be capable of reacting with and altering the function of the contractile proteins. If so, changes in contractile protein function similar to those observed in the present experiment should result.

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