Differential effects of peroxynitrite on contractile protein properties in fast- and slow-twitch skeletal muscle fibers of rat

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Dutka TL, Mollica JP, Lamb GD. Differential effects of peroxynitrite on contractile protein properties in fast- and slow-twitch skeletal muscle fibers of rat. J Appl Physiol 110: 705–716, 2011. First published October 28, 2010; doi:10.1152/japplphysiol.00739.2010.—Oxidative modification of contractile proteins is thought to be a key factor in muscle weakness observed in many pathophysiological conditions. In particular, peroxynitrite (ONOO\(^-\)), a potent short-lived oxidant, is a likely candidate responsible for this contractile dysfunction. In this study ONOO\(^-\) or 3-morpholinosydnonimine (Sin-1, a ONOO\(^-\) donor) was applied to rat skinned muscle fibers to characterize the effects on contractile properties. Both ONOO\(^-\) and Sin-1 exposure markedly reduced maximum force in slow-twitch fibers but had much less effect in fast-twitch fibers. The rate of isometric force development was also reduced without change in the number of active cross bridges. Sin-1 exposure caused a disproportionately large decrease in \(\text{Ca}^{2+}\) sensitivity, evidently due to coproduction of superoxide, as it was prevented by Tempol, a superoxide dismutase mimetic. The decline in maximum force with Sin-1 and ONOO\(^-\) treatments could be partially reversed by DTT, provided it was applied before the fiber was activated. Reversal by DTT indicates that the decrease in maximum force was due at least in part to oxidation of cysteine residues. Ascorbate caused similar reversal, further suggesting that the cysteine residues had undergone \(\text{S}\)-nitrosylation. The reduction in \(\text{Ca}^{2+}\) sensitivity, however, was not reversed by either DTT or ascorbate. Western blot analysis showed cross-linking of myosin heavy chain (MHC) I, appearing as larger protein complexes after ONOO\(^-\) exposure. The findings suggest that ONOO\(^-\) initially decreases maximum force primarily by oxidation of cysteine residues on the myosin heads, and that the accompanying decrease in \(\text{Ca}^{2+}\) sensitivity is likely due to other, less reversible actions of hydroxyl or related radicals. Reversal by DTT indicates that the decrease in maximum force was due at least in part to oxidation of cysteine residues. Ascorbate caused similar reversal, further suggesting that the cysteine residues had undergone \(\text{S}\)-nitrosylation. The reduction in \(\text{Ca}^{2+}\) sensitivity, however, was not reversed by either DTT or ascorbate. Western blot analysis showed cross-linking of myosin heavy chain (MHC) I, appearing as larger protein complexes after ONOO\(^-\) exposure. The findings suggest that ONOO\(^-\) initially decreases maximum force primarily by oxidation of cysteine residues on the myosin heads, and that the accompanying decrease in \(\text{Ca}^{2+}\) sensitivity is likely due to other, less reversible actions of hydroxyl or related radicals.

Contractile apparatus; myosin heads; oxidation; nitrosylation

Oxidative modification of contractile proteins is thought to be a key contributing factor in the muscle weakness observed in many conditions including sepsis (8, 22), inflammatory conditions and rheumatoid arthritis (15, 45), and heart failure and stroke (13, 25, 44). Peroxynitrite (ONOO\(^-\)) is a powerful and toxic oxidant formed when superoxide (\(\text{O}_2^{\bullet-}\)) reacts with nitric oxide (\(\text{NO}^*\)) (see Refs. 4, 32 for review) and has been proposed to be one of the major causes of the muscle weakness observed in many pathophysiological conditions. In healthy cells, superoxide dismutase keeps \(\text{O}_2^{\bullet-}\) concentration ([\(\text{O}_2^{\bullet-}\)]\(\text{i}\)) very low; however, during ischemic events or injury cells can produce large amounts of both \(\text{NO}^*\) and \(\text{O}_2^{\bullet-}\) and can form ONOO\(^-\) in vivo at a rate of 50–100 \(\mu\text{M}/\text{min}\) (3). Despite ONOO\(^-\) being a short-lived molecule [\(\sim100\ \text{ms}\) in cytosol (19, 35)], in pathological states it may be continuously generated for long periods and hence cause substantial oxidative changes to cell function (22). Its production is also considered a trigger of cell death and is known to cause irreversible damage to many target proteins, as well as DNA, and hence contribute to permanent muscle weakness (40).

From intraperitoneal injection of endotoxin in rats and preparations of single intact and skinned diaphragm fibers, the findings of Callahan et al. (7) strongly suggest that the reduction in maximum force observed was due to ONOO\(^-\). In that study, the reduction in maximum force could be prevented either by stopping \(\text{NO}^*\) generation with \(\text{N}^\omega\)-nitro-L-arginine methyl ester (l-NNAME) or by reducing \(\text{O}_2^{\bullet-}\) accumulation with polyethylene glycol-superoxide dismutase. However, it remains unclear whether other oxidants beside ONOO\(^-\) might also have contributed to the observed reductions in maximum force and calcium sensitivity. Direct application of ONOO\(^-\) and 3-morpholinosydnonimine (Sin-1), a compound that decomposes into \(\text{O}_2^{\bullet-}\) and \(\text{NO}^*\), which then combine to form ONOO\(^-\), have been shown previously to reduce maximum force and have differing effects on the \(\text{Ca}^{2+}\) sensitivity of the contractile apparatus in fibers from diaphragm muscle (39). However, diaphragm contains a range of different fiber types (38), and because the fiber types were not determined in that study it remains unknown whether ONOO\(^-\) affects different fiber types in the same way and to the same extent.

Biochemical assays have shown that skeletal muscle S1-myosin ATPase activity is inhibited by both Sin-1 and direct application of ONOO\(^-\) (41). It was concluded that this was due to ONOO\(^-\) predominantly oxidizing cysteine residues on the myosin heads rather than other residues (e.g., methionine or tyrosine). If ONOO\(^-\) oxidizes cysteine residues, the effects of oxidation may be reversible with diethiothreitol (DTT) or other reducing agents in certain circumstances, but this issue has not been closely examined. Tiago et al. (41) used the reducing agent ascorbate to scavenge ONOO\(^-\) and thereby prevent its effects but did not use reducing agents to examine reversibility of the effects. Furthermore, because many of the findings of previous studies (39, 41) are based on the use of Sin-1 to generate ONOO\(^-\) it is unclear whether all of the observed effects were due solely to ONOO\(^-\) or instead to a combination of effects by ONOO\(^-\), \(\text{NO}^*\), \(\text{O}_2^{\bullet-}\), and possibly hydroxyl (\(\text{OH}^*\), which can be formed from \(\text{O}_2^{\bullet-}\)).

Here, using mechanically skinned fibers, we separately examined the effects of applying Sin-1 and ONOO\(^-\) alone on the contractile properties of rat fast-twitch [extensor digitorum longus (EDL)] and slow-twitch [soleus (Sol)] fibers and found major differences in the effects of the two agents and in the susceptibility of the two fiber types. Additionally, we investigated the reversibility of these effects in order to identify the sites of action and molecular mechanisms involved. These findings provide important insight into the actions of ONOO\(^-\)
in muscle fibers that can help identify its role in particular pathophysiological conditions.

MATERIALS AND METHODS

Preparations and force recording. Male Long-Evans hooded rats (~6 mo old) were killed by overdose of fluothane (4% vol/vol) in a glass chamber in accordance with protocols approved by the La Trobe University Animal Ethics Committee. EDL and SOL muscles were rapidly excised and pinned at their resting length under paraffin oil (Ajax Chemicals, Sydney, Australia) in a petri dish lined with Sylgard 184 (Dow Corning, Midland, MI). The muscles were kept cool (~10°C) on an icepack. Segments of individual fibers were mechanically skinned with jeweler’s forceps and mounted at 120% of the resting length on a force transducer (AME801, Hertogen) with a resonant frequency >2 kHz. The skinned fiber segment was first equilibrated for 2 min in a Perspex bath containing 2 ml of a potassium-based “relaxing solution” (see Skinned fiber solutions), and then the contractile apparatus was activated by moving the fiber through a sequence of baths containing progressively higher free [Ca2+]i. Force responses were recorded with a Bioamp pod and PowerLab 4/20 series hardware (ADInstruments, Sydney, Australia). All experiments were performed at room temperature (~23 ± 2°C) or, where specified, at 36°C.

Skinned fiber solutions. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The “relaxing solution” contained 50 mM EGTA (at pCa = −log10 [Ca2+]i (1 > 10)) and the “maximum Ca2+-activating solution” contained 50 mM CaEGTA (pCa 4.5), with each also containing (in mM) 8 total ATP, 36 Na+, 126 K+, 10 phosphocreatine (PCr), and 90 HEPES, at pH 7.1, with total Mg2+ adjusted to give 1 mM free [Mg2+]i (see Refs. 21, 37). These solutions had an osmolality of 295 mOsm/kg H2O. Solutions with intermediate [Ca2+]i were made by appropriate mixture of these two solutions. A relaxing solution at pH 8.5 used for some treatments (see below) was made by adding additional KOH

RESULTS. ONOO− is extremely short-lived in solution at pH ~ 7 (~1 s) (4, 19), its effects were examined not only at pH 7.1 but also at pH 8.5, where its half-life is longer although still only on the order of seconds to tens of seconds. Owing to this short lifetime, these treatments in effect were brief pulsatile applications of ONOO−, and the ONOO− concentrations specified in the text are indicative only of the initial instantaneous peak level (see RESULTS). ONOO− can also be generated from Sin-1, which decomposes in solution to produce NO* and O2·−, which then react together to form ONOO−. On the basis of measurements in Tiago et al. (41), the 500 μM Sin-1 applied here for 20 min at pH 8.5 continuously generated ~5–8 μM ONOO−/min over that interval, and the free level would have remained at an approximately constant, low micromolar level over that period. After exposure to ONOO− or Sin-1, each fiber was washed in standard relaxing solution at pH 7.1 for >20 s. The force-[Ca2+]i relationship was always examined in the standard conditions (pH 7.1) and determined twice in succession before and after every treatment to verify reproducibility. Where treatments involved exposure of the fiber to ONOO− or Sin-1 at pH 8.5, the fiber was always first given the same pH 8.5 exposure (for either 1 or 20 min) without the agent present (see RESULTS).

Contractile apparatus experiments and analysis. The force-[Ca2+]i relationship was determined in each fiber as previously described (28) by exposing the skinned fiber segment to a sequence of solutions heavily buffered at progressively higher free [Ca2+]i (pCa 10–4.5) until maximum force was elicited and the fiber was fully relaxed in the relaxing solution again. This procedure was performed twice before (“control”) and twice after each treatment to verify reproducibility and also gauge any small changes occurring with repeated activation and over time. Force produced at each [Ca2+]i within a given sequence was expressed relative to maximum force generated in that same sequence and analyzed by individually fitting a Hill (nH) curve to each sequence, for each fiber segment, with GraphPad Prism 4 software, yielding separate pCa50 values and nH values (pCa at half-maximum force and Hill coefficient, respectively) for each case. Maximum force reached in each force-[Ca2+]i sequence was expressed relative to that found before any treatment in the given fiber. In some force trace illustrations, the very brief force transients elicited when changing solutions have been truncated for clarity of the records.

Rapid activation of contractile apparatus. A variant of the experiment was used to ascertain the effect of ONOO− treatment on maximum force when force was elicited very rapidly, by applying an effective step rise in free [Ca2+]i. This was achieved by preequilibrating the given fiber segment in a solution with [Ca2+]i buffered at a low level (pCa 7.3) by only a very low concentration of EGTA (100 μM EGTA) and then transferring the fiber to a very heavily Ca2+-buffered solution (50 mM CaEGTA) at pCa 4.5. This method greatly reduces the diffusional delays limiting the rise of [Ca2+]i within the skinned fiber (see Refs. 11, 26), and maximum Ca2+-activated force could be
achieved in ~0.5 s. The fiber was subsequently quickly relaxed in the
standard relaxing solution.

**Rigor force measurements.** Rigor force (force in the absence of
ATP) was examined both before and after ONOO− treatment. The
rigor force was induced with a solution ("MgR") with no ATP or PCr
containing (in mM) 66 HDTA, 90 HEPES, 1.5 total Mg2+ , 126 K+, 36 Na+, and 0.5 EGTA, at pH 7.1; this solution had the same ionic
strength, osmolality, and free [Mg2+ ] as the standard relaxing and
activating solutions. The fiber segment was initially washed for 5–10 s in one bath with MgR solution to remove most ATP and then
transferred into another identical solution until rigor force reached a
plateau level (within 1 min). Cross bridges were then detached by
exposure of the fiber segment once again to the standard ATP-
containing relaxing solution.

**Western blotting of individual fiber segments.** Single Sol fiber
segments (~10 μg wet wt) were analyzed for myosin heavy chain
(MHC I) and TnI protein content by nonreducing Western blotting
using our previously described small-sample methodology (27, 29).
Entire fiber constituents were examined without any fractionation.

Briefly, individual Sol fiber segments in which force measurements
had been made were treated with 5 mM NEM in relaxing solution for
2 min in order to block free sulphydryl sites and then placed in
nonreducing buffer for SDS-PAGE (final concentration: 125 mM Tris
pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 5 mM
NEM). Proteins were separated on 4–20% Criterion Stain-Free Pre-
cast SDS-PAGE gels (Bio-Rad) and then wet transferred to nitrocel-
lulose for 60 min at 100 V in a circulating ice-cooled bath with
transfer buffer containing 140 mM glycine, 37 mM Tris-base, and
20% methanol. Membranes were then probed for MHC I (1:200,
mouse monoclonal, A4.840, Developmental Studies Hybridoma
Bank) and TnI (1:1,000, rabbit polyclonal, Cell Signaling Technol-
yogy) diluted in 1% bovine serum albumin in phosphate-buffered
saline with 0.025% Tween. Chemiluminescent substrate (SuperSignal
West Femto, Pierce) was applied to the membrane, and Western blot
images taken with ChemiDoc XRS fitted with a charge-coupled
device (CCD) camera using Quantity One software (Bio-Rad). With
images taken with ChemiDoc XRS fitted with a charge-coupled
device (CCD) camera using Quantity One software (Bio-Rad). With
the membrane position unchanged, the white light source was
switched on in order to obtain an image of the prestained molecular
weight markers on the membrane, which was then overlaid on the
Western blot image (see Figs. 9 and 10).

**Statistics.** All values are presented as means ± SE, with n denoting
the number of fibers examined. Statistical significance (P < 0.05) was
determined with Student’s two-tailed t-test (paired observations
made on the same fiber, e.g., before vs. after a given treatment). When
comparisons had to be made between different fibers, the two com-
parison treatments were examined alternately on successive fibers
obtained in a given experiment, and significance was tested with
Student’s unpaired t-test.

**RESULTS**

**Effects of ONOO− exposure on contractile properties in Sol and EDL fibers.** In the first set of experiments ONOO− was
directly added to relaxing solution at pH 7.1 and immediately
applied to individual slow-twitch Sol or fast-twitch EDL fibers
in order to gauge its effects on the contractile apparatus, as in
Fig. 1. In slow-twitch Sol fibers a 1-min exposure to a nominal
2.7 mM concentration of ONOO− caused ~20% reduction in
maximum Ca2+-activated force (e.g., Fig. 1A), but the identical
treatment had much less effect in fast-twitch EDL fibers,
causing only ~3% reduction (e.g., Fig. 1B) (mean maximum
force after treatment: 79.6 ± 4.9% and 96.6 ± 1.1% in Sol and
EDL fibers, respectively; Fig. 1E). (Note that even without any
fibers respectively; data not shown, see Ref. 20). Hill curves
individually fitted to the force-pCa data for every staircase in
each fiber (e.g., Fig. 1, C and D) showed that this ONOO−
treatment caused only a small decline in the Ca2+ sensitivity of the
contractile apparatus in Sol fibers (ΔpCa50 = −0.046 ±
0.007, ΔpH−0.6 ± 0.3) and even less in EDL fibers (ΔpCa50 =
−0.015 ± 0.006, ΔpH−0.9 ± 0.5).

Owing to the very short lifetime of ONOO− in solution at
pH 7.1 (~1 s) (19, 35), the ONOO− treatment effectively was
a pulselike application that initially would have reached close
to the added (nominal) concentration but then decayed rapidly
and almost completely in <10 s (see MATERIALS AND METHODS).
In view of this, experiments were also conducted with ONOO−
applied in pH 8.5 solution, where its half-life is extended
although still only on the order of seconds to tens of seconds.
Control experiments showed that exposure to the pH 8.5
conditions for 1 min without any added ONOO− had virtually
no effect on contractile apparatus properties (maximum force:
101 ± 1%, ΔpCa50 −0.008 ± 0.002, and ΔpH−0.0 ± 0.1;
n = 3). Application of ONOO− at pH 8.5 at a nominal
concentration of 2.7 mM caused approximately twice as much
reduction in maximum force in Sol fibers as the same added
concentration at pH 7.1 (Fig. 2), consistent with the ONOO−
persisting for longer and hence exerting a greater net effect, but
possibly also owing to increased reactivity of the sites at pH
8.5 (14, 36). The decrease in Ca2+ sensitivity was also approx-
imately threefold greater when ONOO− was applied at pH 8.5
(ΔpCa50 = −0.118 ± 0.018 and ΔpH−0.6 ± 0.2; n = 4). The
effects of ONOO− increased in a concentration-dependent
manner (Fig. 2) with exposure of Sol fibers to a nominal 4 mM
concentration for 1 min at pH 8.5, reducing maximum force
production by >80% and causing a large reduction in Ca2+
sensitivity (ΔpCa50 approximately −0.2 to −0.3 pCa units; see
Fig. 5). EDL fibers were also affected to a greater extent at
higher concentrations of ONOO−, but always proportionately
less than the effect on Sol fibers at the same concentration.
Even at pH 8.5 the ONOO− evidently had largely or entirely
dissipated over the course of the 1-min treatment period,
because extending the treatment duration had no noticeably
greater effect (data not shown). Furthermore, reaplication of the
ONOO− treatment solution 5 min after its production
showed that it had completely lost its efficacy in reducing
maximum Ca2+-activated force (force after second exposure
98 ± 1% of that beforehand; n = 3, not significantly altered,
P > 0.05).

**Partial reversal of force reduction with DTT and ascorbate
in certain circumstances.** The efficacy of reducing agents in
reversing the effects of ONOO− provides important information
about the types of molecular changes underlying its
actions. In Sol fibers when DTT (10 mM, 10 min) was applied
soon after the 2 mM ONOO− treatment, before the fiber had
been activated, the subsequent activation staircase showed only
a small reduction in maximum force (e.g., Fig. 3A and mean
data in Fig. 3D), indicating that the DTT treatment had par-
tially reversed the effects of the ONOO− treatment on
maximum force production. Interestingly, if the same DTT
treatment was applied after an ONOO−-treated fiber had already
been activated, it had virtually no effect at all in reversing
the loss of force (e.g., Fig. 3, B and C); subsequent treatment with
30 mM DTT for 10 min also had no more effect than treatment
with 10 mM DTT (relative maximum force 100.4 ± 1.8%; n =
The ability of DTT to partially reverse the effects on maximum force in fibers that had not been activated indicates action on cysteine residues (10), and this could have involved reduction of either disulfide bridges or cysteine residues that had undergone \( \text{S-nitrosylation} \) (R-S-NO) or possibly other oxidative changes with the \( \text{ONOO}^- \)/H_2O_2 treatment (2, 32). Ascorbate is claimed to specifically reduce S-nitrosylation, with no effect on disulfide bridges or other oxidized cysteine states (6, 17). Ascorbate treatment (2 mM, 10 min) was found to have effects virtually identical to DTT, partially reversing the reduction in maximum force with \( \text{ONOO}^- \) treatment if and only if it was applied before the fiber was activated (Fig. 3E). Combined successive treatment with DTT and ascorbate was found to be no more effective than either single treatment alone, reversing only about half of the force reduction (2 fibers; not shown).

Fig. 1. Peroxynitrite (\( \text{ONOO}^- \)) causes greater force reduction in slow-twitch fibers. A and B: 2 force-pCa staircases elicited both before and after exposure to \( \text{ONOO}^- \) (nominal concentration of 2.7 mM, pH 7.1 for 1 min) in soleus (Sol; A) and extensor digitorum longus (EDL; B) fibers. pCa of successive solutions: >10, 6.40, 6.22, 6.02, 5.88, 5.75, 5.48, with maximum force at pCa 4.5. Dashed lines highlight maximum force before and after treatment; horizontal arrows indicate force reached at pCa 5.75 in each case, to highlight any sensitivity change. Large force response in pSr 5.2 solution in fiber in A and zero force in B are indicative that the fibers were slow twitch and fast-twitch, respectively (see MATERIALS AND METHODS). C: Hill fits to second force-pCa staircases before and after \( \text{ONOO}^- \) treatment for Sol fiber in A [pCa_{50} and Hill coefficient (n_H) values: 5.93 and 2.9 before, 5.87 and 2.4 after]. D: similar fits for EDL fiber in B (pCa_{50} and n_H: 5.77 and 6.2 before, 5.77 and 5.4 after). E: mean ± SE maximum force in Sol and EDL fibers treated with 2.7 mM \( \text{ONOO}^- \) at pH 7.1 (filled bars) relative to pretreatment level (open bars). *Significantly different from pretreatment level; #EDL data significantly different from Sol data.
the ONOO\(^-\) treatment was so stringent that it reduced maximum force to less than \(~30\%\) (e.g., as with 4 mM ONOO\(^-\) at pH 8.5), neither DTT nor ascorbate was able to appreciably reverse the loss in force (or reduction in Ca\(^{2+}\) sensitivity), irrespective of when they were applied.

**Effects at higher temperature or with GSH present.** In further experiments, maximum Ca\(^{2+}\)-activated force was measured both at 23°C and at 36°C in the same Sol fibers both before and after ONOO\(^-\) treatment (2 mM in 3 fibers and 2.7 mM in 1 fiber, at pH 8.5 and 23°C). Similar to a previous report (37), maximum Ca\(^{2+}\)-activated force was slightly higher (8.9 \(\pm\) 1.7%) at 36°C than at 23°C. The ONOO\(^-\) treatment was found to result in the same relative reduction in maximum Ca\(^{2+}\)-activated force at both temperatures (decreased to 59.3 \(\pm\) 10.4% at 23°C and to 63.6 \(\pm\) 12.3% at 36°C; \(n = 4\), not significantly different, paired \(t\)-test). (Only maximum force was examined in these experiments in order to minimize any possible change occurring with repeated activation, temperature, or time).

GSH is an important intracellular reducing agent, but endogenous GSH is readily diffusible and is lost from a skinned fiber within seconds when the fiber is bathed in a comparatively large pool of solution as in the experiments here (see Refs. 24, 30). When 3 mM GSH was present in the treatment solution with ONOO\(^-\) (2 mM, pH 8.5, 1 min), the reductions in maximum force and Ca\(^{2+}\) sensitivity (maximum force 95.9 \(\pm\) 1.7%, \(\Delta pC_{a50} = 0.040 \pm 0.008\), \(\Delta nH -0.3 \pm 0.1\); \(n = 3\)) were much less than for the same treatment without GSH (75.7 \(\pm\) 2.4%, \(\Delta pC_{a50} = 0.078 \pm 0.013\), \(\Delta nH -0.4 \pm 0.1\); \(n = 12\) Sol fibers). When only 1 mM GSH was present before and during 2.7 mM ONOO\(^-\) treatment at pH 7.1, the reductions in maximum force and Ca\(^{2+}\) sensitivity caused by the ONOO\(^-\) (maximum force 83.4 \(\pm\) 4.8%, \(\Delta pC_{a50} = 0.052 \pm 0.006\), \(\Delta nH -0.9 \pm 0.2\); \(n = 3\) Sol fibers) were not significantly different from those when GSH was absent (79.6 \(\pm\) 4.9%, \(\Delta pC_{a50} = 0.046 \pm 0.007\), \(\Delta nH -0.6 \pm 0.3\); \(n = 9\) Sol fibers).

**Effect of Sin-1 exposure on contractile properties.** The effect of ONOO\(^-\) on the contractile apparatus was also examined by using Sin-1 to generate continuous low levels of ONOO\(^-\). Sol and EDL fibers were exposed for 20 min to a solution at pH 8.5 containing 500 \(\mu\)M Sin-1, which based on the measurements of others would have contained ONOO\(^-\) at an approximately steady, low micromolar level over that whole period (see MATERIALS AND METHODS). The constancy in level over the treatment period was further indicated by the fact that the magnitude of its effect on contractile properties (described below) appeared to increase in direct proportion with the duration of exposure up to 20 min (data not shown).

An initial exposure to pH 8.5 conditions for 20 min itself caused some change in the contractile properties: maximum force decreased to 91 \(\pm\) 2% of initial and pCa\(_{50}\) decreased \(-0.038 \pm 0.009\) and \(nH -0.2 \pm 0.1\) in the four Sol fibers examined. However, a subsequent 20-min exposure to these same conditions caused little further change (\(~5\%\) and \(~4\%) reductions in maximum force, with \(\Delta pC_{a50} = -0.013\) and no alteration in \(nH\)). Consequently, all fibers in this series of experiments were first subjected to a control exposure to pH 8.5 conditions for 20 min, and two force-pCa staircases were elicited, before examination of the effects of exposure to Sin-1, thereby avoiding virtually all of the compounding effects of the pH 8.5 exposure itself.

As occurred with direct application of ONOO\(^-\) (Fig. 1), exposure to Sin-1 for 20 min caused a substantial reduction in maximum Ca\(^{2+}\)-activated force in slow-twitch Sol fibers and only a comparatively small reduction in fast-twitch EDL fibers (Figs. 4, A and C; maximum force after treatment 72 \(\pm\) 2% and 95 \(\pm\) 1% of initial in Sol and EDL fibers, respectively). However, Sin-1 treatment also caused a very large reduction in Ca\(^{2+}\) sensitivity in every Sol fiber, with little change in steepness of the relationship (\(nH\)) (e.g., Fig. 4B), and again much less effect in the EDL fibers (\(\Delta pC_{a50} = -0.349 \pm 0.023\) and \(\Delta nH -0.2 \pm 0.1\) in 14 Sol fibers; \(\Delta pC_{a50} = -0.070 \pm 0.007\) and \(\Delta nH -0.5 \pm 0.1\) in 9 EDL fibers; \(P < 0.05\) in all cases). The extent of the reduction in Ca\(^{2+}\) sensitivity for a given reduction in maximum force was approximately four times greater with Sin-1 treatment than with direct ONOO\(^-\) application at the same pH (Fig. 5), indicative of substantial differences in the molecular actions of the two treatments. Similarly, in a Sol fiber treated at pH 7.1 with 8 mM Sin-1 for 20 min, maximum Ca\(^{2+}\)-activated force was reduced by 15% and the Ca\(^{2+}\) sensitivity of the contractile apparatus (\(\Delta pC_{a50} = -0.131\) and \(\Delta nH -0.7\)) was again proportionately more decreased than with direct ONOO\(^-\) treatment at the same pH.

Sin-1 generates ONOO\(^-\) by producing both O\(_2^-\)* and NO*, and so the question arises as to whether the effects of Sin-1 treatment were due to ONOO\(^-\) or to the other radicals, or even to some other unrelated effect of Sin-1. This was investigated by applying Sin-1 together with Tempol, a potent superoxide dismutase mimetic (12), in order to remove most superoxide and also reduce consequent generation of ONOO\(^-\). Strikingly, the copresence of Tempol prevented most of the effects of the Sin-1 treatment (Fig. 6A), markedly attenuating the reduction in maximum force (Fig. 6B) and, to an even greater extent, the reduction in Ca\(^{2+}\) sensitivity (Fig. 5 and Fig. 6C) (after treatment of Sol fibers with Sin-1 with Tempol: maximum force 90 \(\pm\) 1%, \(\Delta pC_{a50} = -0.052 \pm 0.013\), \(\Delta nH -0.3 \pm 0.1\); \(n = 5\)). As expected, the copresence of Tempol (0.5 mM) did not prevent the decrease in force and Ca\(^{2+}\) sensitivity occurring with direct ONOO\(^-\) application (2 mM, pH 8.5) (maximum force 63.1 \(\pm\) 10.9%, \(\Delta pC_{a50} = -0.117 \pm 0.024\); \(n = 3\), not significantly different from ONOO\(^-\) alone). These data indicate that the effects of Sin-1 were dependent in large part on...
the generation of O$_2$•$^-$ and were not due to some nonspecific action of Sin-1 itself (see DISCUSSION for further implications).

The ability of DTT to reverse the effects of Sin-1 treatment was also examined. Similar to the ONOO$^-$ experiments, when DTT (10 mM, 10 min) was applied before fiber activation it partially reversed the reduction in maximum force (Fig. 6D), but it had no effect on the reduction in Ca$^{2+}$ sensitivity (Fig. 6E) (maximum force 82 ± 2%, ΔpCa$_{50}$ −0.311 ± 0.038 and ΔnH −0.4 ± 0.1 in 8 Sol fibers exposed to Sin-1 and then DTT; 71 ± 3%, −0.339 ± 0.017, and −0.1 ± 0.1, respectively, in 8 paired Sol fibers without DTT). Again, as with direct ONOO$^-$ treatment, DTT had no effect when applied after fiber activation (maximum force 70 ± 7%, ΔpCa$_{50}$ −0.320 ± 0.040, and ΔnH −0.2 ± 0.1 after Sin-1 treatment and 72 ± 6%, −0.314 ± 0.040, and −0.4 ± 0.2, respectively, in 5 Sol fibers after subsequent DTT treatment, all relative to original pretreatment level).

**Effect of ONOO$^-$ on rate of force development and cross bridge formation.** Step rises in [Ca$^{2+}$] were used to elicit rapid force activation in order 1) to verify that the effect of ONOO$^-$ treatment on force production was not influenced by the relatively long time taken to elicit the normal force-pCa “staircase” (~1 min) and 2) to gauge the effect of ONOO$^-$ treatment on the rate of force development. A large steplike change in free [Ca$^{2+}$] was applied to each skinned fiber by rapidly substituting a very weakly buffered solution at pCa 7.3 with a very heavily Ca$^{2+}$-buffered solution at pCa 4.5 (Refs. 11, 26; see MATERIALS AND METHODS). The extent of reduction of maximum force after exposure to ONOO$^-$ (2 mM, at pH 8.5) was found to be the same irrespective of the rate of force activation, even for the first rapid activation episode (e.g., Fig. 7A). With these fast [Ca$^{2+}$] steps the time taken for force to rise from 10% to 90% of its final level was increased by 8.0 ± 1.2% after the ONOO$^-$ treatment in the four Sol fibers examined, and the rate of isometric force development was reduced by 26 ± 2% (Fig. 7, B and C); in these same fibers maximum force was reduced by 21.5 ± 3.1%. The exposure to pH 8.5 conditions itself had no significant effect on the rate of force development (103 ± 2% of preexposure level; Fig. 7C).

Rigor force development was used to gauge whether ONOO$^-$ treatment affected the number of cross bridges contributing to force development (34, 37). In the five Sol fibers examined (e.g., Fig. 8), rigor force was unchanged in absolute terms by ONOO$^-$ treatment (2 mM, pH 8.5), being 36 ± 4%
of the initial maximum force level before ONOO− treatment and 34 ± 5% of that same initial level after treatment (P > 0.05, not significant), even though maximum force was reduced to 74 ± 2% of initial by this treatment.

Additional findings. The difference in effect of ONOO− on Sol and EDL fibers was not attributable to differences in fiber diameter. The level of reduction in maximum Ca2+-activated force had no association with fiber diameter (correlation index $r^2 = 0.07912$). This was tested by deliberately “overskinning” or splitting some fibers in order to give a large range of diameters. In other experiments it was further found that the effects of Sin-1 treatment were no different when skinned fibers were first treated with Triton X-100 to remove intracellular membranes (see MATERIALS AND METHODS); 500 μM Sin-1 exposure still reduced maximum force (to 96% and 92% in EDL fibers and to 71% and 70% in Sol fibers) and Ca2+ sensitivity ($\Delta p_{Ca_{50}}$ 0.068 and −0.061 and −0.333 and −0.297, respectively), with the changes being indistinguishable from those without Triton treatment.

(See Fig. 5, as in Figs. 1 and 4, Linear regression line of best fit is shown for each data set. Mean data (+SE in both dimensions) are also shown for other Sol fibers when 0.5 mM Tempol was present with the Sin-1, as in Fig. 6. (Figs. 4–6). Finally, after maximum force had been reduced by ONOO− treatment (2 mM, pH 8.5), it remained unchanged when exogenous CPK was added to the bathing solution (30 units of activity/ml) (maximum force: 78 ± 4% and 77 ± 4% of initial force, before and after CPK addition, respectively). This level of exogenous CPK was shown previously to be sufficient to ameliorate any changes due to CPK dysfunction (see Ref. 11), indicating that the effects of ONOO− treatment on the contractile apparatus were not due to disruption of endogenous CPK. Furthermore, the effects reported here were also not caused by breakdown products of ONOO− (e.g., nitrite, nitrate, or carbonate), as reapplication of the same solution 5 min after decay had no effect whatsoever.

Western blotting of MHC I and TnI in individual fiber segments. Finally, we sought to use Western blotting to examine whether the ONOO− treatment caused S-nitrosylation of cysteines on the myosin heads, which has been shown to inhibit actin-activated myosin ATPase activity (31). However, this was not possible because it was found that the ONOO− treatment caused extensive cross-linking of MHC into much larger protein complexes (Fig. 9, bottom), dispersing the myosin in such a way that it precluded any quantitative examination of myosin nitrosylation. Treatment with DTT and/or ascorbate reduced but did not fully prevent the cross-linking seen with Western blotting, irrespective of whether these agents were applied before or after fiber activation. Western blotting for actin showed that it was not involved in this myosin cross-linking (not shown). Western blotting was also used to examine whether the ONOO− treatment might have caused cross-linking of TnI to actin and thereby interfered with normal Ca2+ activation of force. As seen in Fig. 10, TnI cross-linking occurred to only a very small extent in ONOO−-treated fibers, and the amount detected showed no relationship at all with the decrease in maximum force occurring in the given fiber segment.
DISCUSSION

Slow-twitch fibers are more susceptible to ONOO\(^{-}\) than fast-twitch fibers. One major finding of the present study was that slow-twitch fibers are much more susceptible to ONOO\(^{-}\) than fast-twitch fibers. At both pH 7.1 and pH 8.5, exposure to ONOO\(^{-}\) reduced maximum force in slow-twitch Sol fibers in a concentration-dependent manner, with the Ca\(^{2+}\) sensitivity of the contractile apparatus being far less affected (Fig. 1). Similar to ONOO\(^{-}\) treatment, exposure to Sin-1 (an ONOO\(^{-}\) donor) also caused much greater reduction in maximum force in slow-twitch Sol fibers than in fast-twitch EDL fibers (Fig. 4C). These differences between EDL and Sol fibers were not attributable to any differences in fiber diameter (see RESULTS). Interestingly, another oxidant, H\(_2\)O\(_2\), has also been found to cause greater reductions in maximum force in Sol fibers than in EDL fibers (~22% and ~13%, respectively; rat skinned fibers) (34). Furthermore, we found a similar fiber type difference in susceptibility in skinned fibers exposed to OH\(^{\bullet}\) produced via the Fenton reaction during exposure to H\(_2\)O\(_2\) in the presence of myoglobin, with maximum force reduced to a greater extent in Sol fibers than in EDL fibers at every [H\(_2\)O\(_2\)] and exposure time examined (28).

Significantly, intraperitoneal injections in rats of endotoxin, which putatively exerts its effects in muscle by generating ONOO\(^{-}\) intracellularly (7) (see introduction), was found to cause greater reductions in force in Sol fibers (by ~28%) than in EDL fibers (by ~22%) (38). That finding must reflect the net outcome of any differences between the muscles in a number of critical factors, such as the rate and amount of reactive oxygen species (ROS)/reactive nitrogen species (RNS) production, susceptibility of the contractile apparatus, and intracellular antioxidant levels, etc. The much greater susceptibility of slow-twitch Sol fibers to ONOO\(^{-}\)-induced changes found here likely had a major bearing on the overall effect. Slow-twitch fibers have been found to have approximately fivefold more of the endogenous antioxidant GSH than fast-twitch fibers (18). Given the finding here that 3 mM GSH largely prevented the ONOO\(^{-}\)-mediated reduction in maximum force, it seems likely that this level of GSH would help mitigate problems...
arising from the heightened sensitivity of slow-twitch fibers to oxidant-induced damage, as well as perhaps helping to counter any issue with their greater density of mitochondria and possibly greater resultant O$_2^{ullet-}$ production. However, the concentration of GSH in vivo is not static and can become depleted with prolonged exposure to oxidative stress, and it was found here that the presence of 1 mM GSH did not significantly alter the ONOO$^-$-mediated decrease in either maximum Ca$^{2+}$-activated force or Ca$^{2+}$ sensitivity of the contractile apparatus (∼83% maximum force and ΔpCas$_{50}$ approximately −0.052 pCa units with 1 mM GSH present vs. 80% and ΔpCas$_{50}$ approximately −0.046 pCa units without GSH). Thus ONOO$^-$ is likely to exert deleterious effects on contraction in certain circumstances, particularly with local high levels or continuous low levels for a prolonged period.

The reduction in maximum Ca$^{2+}$-activated force found here with ONOO$^-$ exposure was accompanied by an ∼25% slowing in the rate of isometric force development without any apparent change in the total number of active cross bridges (Figs. 7 and 8). As actin-activated ATPase activity in isolated myosin heads has been shown to be inhibited by ONOO$^-$ over a range comparable to that used here (41), and also by other oxidants and nitrosylating agents such as sodium nitroprusside, S-nitrosoglutathione (GSNO), and NEM (Refs. 31, 33 and references therein), it seems most likely that the reductions in the peak and rate of force production seen in the skinned fibers here were due to ONOO$^-$-induced inhibition of the myosin ATPase. The ATPase activity of the myosin head is known to be readily inhibited by oxidation of two particular reactive cysteine residues, Cys707 and Cys697, referred to as SH$_1$ and SH$_2$, respectively. It seems somewhat surprising then that Sol fibers are far more susceptible to oxidant-induced dysfunction because the amino acid sequence between SH$_1$ and SH$_2$ is identical in slow- and fast-twitch myosin, although some adjacent residues do differ, and possibly this could have large effects on the accessibility or reactivity of one or both of these sites. Alternatively, it is possible that the difference in susceptibility of Sol and EDL fibers might result from some difference in the endogenous state of the target sites, such as glutathionylation, or involve oxidation of sites other than SH$_1$ and SH$_2$ (31).

Reversible and irreversible reductions in maximum force. The loss in maximum force caused by ONOO$^-$ was partially reversed upon treatment with either DTT or ascorbate, provided that the reduction was not too great (e.g., force decrease <50%) and the reducing agent was applied before the fiber was activated. The fact that DTT treatment was able in some circumstances to reverse a large portion of the decrease in maximum force caused by the ONOO$^-$ treatment implies that...
The decreased force was due in part to oxidative change involving cysteine residues (10), consistent with the findings on isolated myosin heads (see above, Ref. 41). The additional finding here that ascorbate, an agent that putatively only reduces S-nitrosylation (6, 17), had effects virtually identical to those of DTT further suggests that the cysteines had undergone S-nitrosylation (i.e., RSNO) rather than forming disulfide bridges (i.e., RSSR) or undergoing other oxidative changes. RNS and ROS can cause S-nitrosylation and sulfenation (i.e., RSOH) of cysteine residues (see Ref. 1 for review), but more prolonged treatment can lead successively to sulfination (RSO2H) and then to sulfonation (RSO3H), the latter considered largely irreversible (see also Ref. 9). Such transition to sulfination and finally through to sulfonation might explain why the reduction in maximum force with more stringent ONOO− treatments (i.e., maximum force decreased by ~50% or more) was not reversible with either DTT or ascorbate irrespective of when they were applied. Alternatively or additionally, the more stringent ONOO− treatments may have oxidized residues other than cysteine (e.g., methionine and tyrosine), which would not have been reversible with either DTT or ascorbate in these in vitro skinned fibers (10, 14); in intact cells in vivo methionines can be reduced by the enzyme methionine sulfoxide reductase (14). Oxidation of methionine and tyrosine residues might also explain the observation of
myosin cross-linking to form large protein complexes that could not be fully reversed with DTT or ascorbate (Fig. 9). It is important to note that the substantial level of myosin cross-linking seen in the Western blots may have predominantly occurred when the fiber segments were solubilized in non-reducing buffer rather than when the myosin was still in its normal position in the sarcomeric lattice, particularly if the fiber had not been activated. Irrespective of when it occurred, the myosin cross-linking is clear evidence that the ONOO⁻ treatment had caused extensive oxidation of the fiber proteins.

Contractile activation can cause irreversible changes. An interesting further observation was that the oxidative changes to maximum force were only reversible if the fiber had not been activated. This observation helps explain the earlier finding that DTT was unable to reverse the force reduction occurring in skinned fibers from diaphragm muscle treated with endotoxin (7), because those fibers were activated before the effect of DTT was examined. Similarly, DTT was previously found to poorly reverse ONOO⁻-induced decline in force in cardiomyocytes, and again those preparations were activated before DTT was applied (16); interestingly, DTT was found in this latter study to fully reverse the level of cysteine oxidation, but this was concluded from parallel measurements made on isolated myofilament proteins rather than on the filaments subjected to the activation procedure. The activation-dependent loss of reversibility of oxidative changes reported here mirrors the findings of the study in Ref. 43, which examined mechanically skinned fibers from EDL and peroneus longus muscles exposed to elevated temperatures (e.g., ≥40°C). In that study, the reduction in maximum force in the skinned fibers was only reversed if DTT was applied before activation. The study concluded that the reduction in force was associated in some way with O₂⁻⁻, as application of a O₂⁻⁻ scavenger, Tiron, ameliorated the effect. Additionally, the study found that the slow-twitch fibers in the peroneus longus muscle were more deleteriously affected than the fast-twitch EDL fibers even with the O₂⁻⁻ scavenger present. Taken together, those findings on whole muscles stimulated at elevated temperatures exhibit striking similarities to the present findings on the effect of ONOO⁻, strongly suggesting that the force reduction occurring in the heated muscles was due to ONOO⁻ formation in the muscles that acted on cysteine residues in the myosin heads.

The reason why activation of a preparation renders the changes irreversible by DTT or ascorbate could be that the contraction brings other noncysteine oxidized residues (e.g., methionine), on myosin or neighboring proteins, together and leads to the formation of an irreversible cross-link. Alternatively, activation might favor “unfolding” of oxidized myosin heads, which is irreversible and renders them dysfunctional (41).

Reduced Ca²⁺ sensitivity of contractile apparatus. In addition to its effects on the rate and peak of force production, Sin-1 exposure also caused a disproportionately larger decrease in the Ca²⁺ sensitivity of the contractile apparatus than that occurring with directly applied ONOO⁻ (Figs. 4 and 5), and this was evidently due to the coproduction of O₂⁻⁻ or some derivative because the effect was blocked with Tempol (Fig. 6). Mild ONOO⁻ exposure caused relatively little reduction in Ca²⁺ sensitivity, and it was only when the ONOO⁻ treatment was more stringent (i.e., 4 mM causing an ~80% reduction in maximum force) that the Ca²⁺ sensitivity was very substantially reduced (ΔpCa₅₀ approximately −0.2 to −0.3 pCa units; see Fig. 5). In contrast to the partial reversal of maximum force reduction occurring with mild ONOO⁻ treatment, the accompanying reduction in Ca²⁺ sensitivity was not reversible; in fact, the reduction in Ca²⁺ sensitivity occurring in the various circumstances examined was never found to be reversible with DTT or ascorbate. The most likely explanation for these findings is that the two effects are caused by different processes, with the reduction in maximum force being caused primarily by ONOO⁻ and the reduction in Ca²⁺ sensitivity being caused by O₂⁻⁻, or more likely its derivative, OH⁺. The latter is suggested by our previous findings (28) that 1) OH⁺ exposure causes relatively large decreases in Ca²⁺ sensitivity for a given decrease in maximum force (i.e., in Sol fibers ΔpCa₅₀ approximately −0.27 with maximum Ca²⁺-activated force reduced by ~39%), very much like that found here with Sin-1, and 2) application of Tempol did not prevent that effect, indicating that it was likely due directly to OH⁺ rather than to some possible associated generation of O₂⁻⁻. In the present study, when fibers were exposed to a mixture of Sin-1 and Tempol the large decline in Ca²⁺ sensitivity was almost entirely prevented (Fig. 6), as expected because the removal of both O₂⁻⁻ and the associated ONOO⁻ would have greatly decreased any generation of OH⁺ radicals. ONOO⁻ is capable of generating OH⁺ directly upon decomposition (40), and thus a larger [ONOO⁻] would generate a higher [OH⁺] and this could account for the progressively larger reduction in Ca²⁺ sensitivity observed at higher [ONOO⁻].

Concluding remarks. This study shows that slow-twitch Sol fibers are far more susceptible to the oxidative actions of ONOO⁻ than fast-twitch EDL fibers. Furthermore, it appears that the myosin heads are more affected than other contractile proteins and that critical cysteine residues on the myosin heads undergo S-nitrosylation, which reduces maximum force. This study also highlights that caution is required when using Sin-1 to examine ONOO⁻-specific effects, as Sin-1 also had additional O₂⁻⁻-mediated effects unrelated to ONOO⁻. Furthermore, reversibility or irreversibility of oxidative changes can be dependent on whether or not the preparation has been activated before application of the reversing agent. Finally, the findings here provide important insight into the actions of ONOO⁻ in muscle fibers, helping delineate its role in various pathological conditions.

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REFERENCES

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