Nitric Oxide Protects the Skeletal Muscle Ca\textsuperscript{2+} Release Channel from Oxidation Induced Activation*

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Reactive oxygen intermediates and nitric oxide modulate the contractile function of skeletal muscle fibers, possibly via direct interaction with the Ca\textsuperscript{2+} release channel. Oxidants produce disulfide bonds between subunits of the Ca\textsuperscript{2+} release channel tetramer, and this is accompanied by an increase in channel activity. The sulphydryl alkylating agent N-ethylmaleimide has three distinct effects on Ca\textsuperscript{2+} release channel activity: first, channel activity is decreased (phase 1); then with continued exposure the activity is dramatically increased (phase 2); and finally, the channel is again inhibited (phase 3) (Aghdasi, B., Zhang, J. Z., Wu, Y., Reid, M. B., and Hamilton, S. L., (1997) J. Biol. Chem. 272, 3739–3749). Both H\textsubscript{2}O\textsubscript{2} and nitric oxide (NO) block the phase 1 inhibitory effect of N-ethylmaleimide. NO donors, at concentrations that have no detectable effect on channel activity, block intersubunit cross-linking and prevent activation of the channel by the disulfide inducing agent, diamide. These findings support a model in which NO modulates the activity of the Ca\textsuperscript{2+} release channel by preventing oxidation of regulatory sulfhydryls. However, higher concentrations of NO donors activate the channel and produce intersubunit cross-links, supporting a bifunctional effect of NO on channel activity. Low NO concentrations prevent oxidation of the Ca\textsuperscript{2+} release channel whereas higher concentrations oxidize it.

Nitric oxide (NO\textsuperscript{1}; including NO\textsuperscript{+}, NO\textsuperscript{−}, and NO\textsuperscript{2}) and reactive oxygen intermediates (ROI) are produced in skeletal muscle and modulate excitation-contraction coupling (3, 4). One model to explain these effects is that endogenous NO and ROI influence calcium homeostasis (5) by directly altering the activity of the skeletal muscle Ca\textsuperscript{2+} release channel (RYR1). Favero et al. (6) reported a biphasic effect of H\textsubscript{2}O\textsubscript{2} on skeletal muscle RYR1; first it activated and then it inhibited the channel. Activation of the channel with H\textsubscript{2}O\textsubscript{2} was reversible with reducing agents such as dithiothreitol (DTT). These workers proposed that the increase in channel activity is due to the formation of cross-links between the RYR1 and triadin (6, 7). We (1) demonstrated that diamide produces disulfide bonds between subunits of the RYR1 tetramer and proposed that this cross-linking activates the channel. In these studies we also demonstrated that alkylation of RYR1 by N-ethylmaleimide (NEM) has triphasic effects on single channel activity and on \textsuperscript{3}Hryanodine binding. Alkylation of hyperactive (phase 1) sulfhydryls and oxidative cross-linking of sulphydryls are mutually exclusive processes; either can occur but not both.

NO generating compounds have also been shown to alter the activity of RYR1. The exact nature of the effects of NO compounds on RYR1 is, however, controversial. Mezsáros et al. (8, 9) observed inhibition of the RYR1 channel activity by NO donors, whereas Stoyanovsky et al. (10, 11) found that NO donors activate RYR1. Both groups postulated that these effects are mediated by the reaction of NO with sulphydryls on the Ca\textsuperscript{2+} release channel. The divergent effects of NO on Ca\textsuperscript{2+} release channel could arise from differences in 1) the reaction conditions, 2) the amount of NO generated, or 3) the reaction by-products.

ROI enhance contractile function, whereas NO opposes this effect (3–5). In this study we tested the hypothesis that oxidants and NO interact directly with RYR1; oxidants activate the channel, and NO blocks the oxidative activation. Diamide cross-linking and NEM alkylation are used as tools to elucidate the mechanism of interaction of oxidants and NO with the functional sulphydryls of RYR1.

EXPERIMENTAL PROCEDURES

Materials—[\textsuperscript{3}H]Ryanodine (68.30 Ci/mmol) was purchased from NEN Life Science Products. Ryanodine was obtained from Calbiochem. Diamide, NEM, and DTT were obtained from Sigma. S-nitroso-N-acetyl-\textit{t}-penicillamine (SNAP), PAPA NONOate (NOC-15) and MAMA NONOate (NOC-9) were purchased from Alexis Corporation (San Diego, CA). Phosphatidylethanolamine (bovine heart) and phosphatidylcholine (bovine brain) were obtained from Avanti Polar-Lipids, Inc. (Alabaster, AL). Ultima Gold scintillation was purchased from Packard (Meriden, CT).

Sarcoplasmic Reticulum (SR) Membrane Preparation—SR membranes were prepared from rabbit backstrap and hindleg skeletal muscle and purified by using sucrose gradient centrifugation as described elsewhere (12, 13). Protein was estimated by the method of Lowry et al. (14), using bovine serum albumin as standard.

Equilibrium \textsuperscript{3}Hryanodine Binding—\textsuperscript{3}Hryanodine was incubated overnight (15–17 h) at room temperature (23 °C) with 5–75 μg of SR membranes in 100–250 μl of buffer containing 0.3 M NaCl, 50 mM MOPS (pH 7.4), 100 μg/ml bovine serum albumin, 0.1% CHAPS, 100 μM free CaCl\textsubscript{2}, 100 μM phenylmethylsulfonyl fluoride, 200 μM aminobenzamidine, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. Nonspecific binding was defined in the presence of either 10 or 100 μM ryanodine. Bound \textsuperscript{3}Hryanodine was separated from free by rapid filtration of the sample through Whatman GF/F glass fiber filters followed by 5 × 3-ml washes with ice-cold wash buffer containing 0.3 M NaCl, 100 μM CaCl\textsubscript{2}, and 10 mM MOPS (pH 7.4). The radioactivity bound to the filters was quantified by liquid scintillation counting of the filters in 5 ml of Ultima Gold scintillant.

Alkylation of SR membranes with NEM—0.5-mg SR membranes were treated with and without NO donors or H\textsubscript{2}O\textsubscript{2} at room temperature for 20 min. The membranes are then pelleted in a Beckman Airfuge and resuspended in binding buffer. After removing 20-μg samples for con-
RESULTS

H$_2$O$_2$ Blocks Phase 1 NEM Alkylation and Produces Intersubunit Cross-links within the RYR1 Tetramer—We have previously demonstrated that diamide blocks phase 1 inhibition of the channel by NEM and produces disulfide bonds between neighboring subunits within the RYR1 tetramer. In the present study, we asked whether the physiological ROI, H$_2$O$_2$, could produce the same changes that were observed with diamide. SR membranes were treated with H$_2$O$_2$, washed, alkylated with NEM for different periods of time, reduced with DTT, and assayed for ability to bind [3H]ryanodine. Similar to diamide pretreatment, H$_2$O$_2$ pretreatment prevented NEM under phase 1 conditions from inhibiting [3H]ryanodine binding (Fig. 1A). H$_2$O$_2$ had no significant effect on the phase 2 and 3 reactions. Again similar to diamide, H$_2$O$_2$ produced intersubunit cross-links that are reversed with DTT (Fig. 1B). For comparison, this figure also shows the dimer formation by diamide. In the membranes used for these experiments, RYR1 (Fig. 1B, band a) was partially proteolyzed by endogenous calpain to produce a 410-kDa fragment (band b), which is missing an N-terminal domain (16, 17). Dimers of both the full-length RYR1 and the 410-kDa fragment are observed. As previously discussed (1), we do not detect any cross-linking of the full-length 565-kDa band to the 410-kDa band, suggesting that when cleavage at the N-terminal site occurs, all subunits within a tetramer are cleaved. The dimers can be reduced to monomers by treatment with DTT. Diamide cross-linking of RYR1 is associated with an increase in the activity of the channel (1, 18). H$_2$O$_2$ also activates the channel reconstituted into planar lipid bilayer (Fig. 1C). The steady-state open probability ($P_\text{O}$) of the channel increased from 0.014 ± 0.003 to 0.312 ± 0.075 ($p < 0.05$) with the addition of 5 mM H$_2$O$_2$ ($n = 3$) and decreased to 0.077 ± 0.057 ($p < 0.05$) with the addition of 10 mM DTT ($n = 3$) after H$_2$O$_2$. To determine the time constants, the open- and closed-time histograms were fit with two exponential equation described under “Experimental Procedures”.

FIG. 1. H$_2$O$_2$ blocks phase 1 of alkylation, induces intersubunit cross-links and increases single channel activity. A, SR membranes (0.4–0.6 mg) were treated with 5 mM H$_2$O$_2$ for 10 min at room temperature. These membranes were then treated with 5 mM NEM as described under “Experimental Procedures”; (C) represents the alkylation curve for H$_2$O$_2$ pretreated membranes and (●) is the control alkylation curve with no H$_2$O$_2$ pretreatment. Data shown as mean ± S.E. (n = 3). B, 5% SDS-polyacrylamide gel electrophoresis showing intersubunit cross-links induced by H$_2$O$_2$ (a, 565 kDa RYR1; b, 400 kDa). Lane 1 shows RYR1 on non-reducing gel when the membranes were not treated with H$_2$O$_2$ (A, 2 × 565 kDa RYR1; B, 2 × 400 kDa). Lane 2 shows the SR membranes that were pretreated with 5 mM H$_2$O$_2$ on a non-reducing gel. Lane 3 shows the SR membranes pretreated with 500 μM diamide for comparison with H$_2$O$_2$. Lanes 4–6 show electrophoresis data for H$_2$O$_2$ and DTT treatment of RYR1. C, Single channel tracings of RYR1 reconstituted into a planar lipid bilayer showing the effect of H$_2$O$_2$ on channel activity. Trace 1 represents control records of RYR1 reconstituted in planar lipid bilayer (O and C reflect the open- and closed-state of the channel, respectively). Trace 2 shows the effect of the addition of 5 mM H$_2$O$_2$ to the cis chamber, and trace 3 shows the effect of the addition of 10 mM DTT to the cis chamber 10 min after the addition of H$_2$O$_2$. 

FIG. 2. NO donors block phase 1 of alkylation. A–C, SR membranes (0.4–0.6 mg) were treated with 100–500 μM SNAP, NOC-15, or NOC-9 for 20 min at room temperature. After this treatment, alkylation with 5 mM NEM was performed as described under “Experimental Procedures.” Panels 1–3 represent nine independent experiments (three for each compound) done with SNAP, NOC-15, and NOC-9, respectively. Filled circles (●) represent alkylation of contemporary controls, and open circles (○) represent alkylation after NO donor pretreatment. D, this figure shows the effect of by-products of NO donors on alkylation. NO donors were left at room temperature at pH 5.74 for at least 72 h; SR membranes (0.4–0.6 mg) then were treated with 100–500 μM of NO-depleted products for 20 min at room temperature. After this alkylation was performed with 5 mM NEM as described under “Experimental Procedures.” The filled circles (●) show control alkylation with 5 mM NEM; (■), (▲), and (▼) show alkylation in the presence of SNAP, NOC-15, and NOC-9 by-products, respectively. Data shown as mean ± S.E. (n = 3).

TABLE 1
Effect of H2O2 on single channel activity

<table>
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<tr>
<th></th>
<th>P_o</th>
<th>τ_1</th>
<th>τ_2</th>
<th>τ_3</th>
<th>τ_4</th>
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<tr>
<td>Control</td>
<td>0.014 ± 0.003</td>
<td>0.21 ± 0.03</td>
<td>1.22 ± 0.22</td>
<td>10.41 ± 3.14</td>
<td>1.75 ± 0.14</td>
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<tr>
<td>5 mM H2O2</td>
<td>0.312 ± 0.075^a</td>
<td>0.38 ± 0.03^a</td>
<td>3.35 ± 0.41^a</td>
<td>41.23 ± 3.18^a</td>
<td>1.34 ± 0.17^a</td>
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</table>

^a P < 0.05, Student’s paired t test was used to determine the significance of change. τ_1, component 1 of time-constant for open state; τ_2, component 2 of time-constant for open state; τ_3, component 1 of time-constant for closed state; τ_4, component 2 of time-constant for closed state; P_o, steady-state probability of opening.
The result of these fits are summarized in Table I. These findings are in agreement with those of Favero et al. (6) with RYR1 and Boraso et al. (19) with RYR2.

NO Donors Block Phase 1 of NEM Alkylation—We next examined the effects of NO donors on the three phases of NEM reaction with RYR1. Figs. 2, A-C show the effects of SNAP, NOC-9, and NOC-15 on the three phases of alkylation. Membranes were preincubated with the NO donor, washed, and then reacted with NEM. Each NO donor blocked phase 1 inhibition by NEM and had little or no effect on phase 2 and 3. To demonstrate that the blockage of phase 1 alkylation was due to nitric oxide, each NO donor was incubated at room temperature for 50–500 times its half-life to deplete NO. The NO-depleted compounds were then added to SR membranes prior to alkylation with 5 mM NEM. As shown in Fig. 2D the by-products of SNAP, NOC-15, and NOC-9 did not block phase 1 NEM inhibition of [3H]ryanodine binding.

Effect of NO Donors on Intersubunit Cross-linking—To determine if NO donors produce intersubunit cross-links, we treated SR membranes with NOC-9, NOC-15, and SNAP. At concentrations that blocked the phase 1 alkylation, none of these reagents produced higher molecular weight complexes of RYR1. However, exposure of SR membranes to NO donors prevented cross-linking of the subunits during subsequent diamide treatment. Fig. 3A shows that SNAP pretreatment greatly reduced the ability of diamide to produce intersubunit cross-links; NOC-9 and NOC-15 produced similar results (data not shown). Fig. 3B summarizes the blockage of intersubunit

![Figure 3](http://www.jbc.org/)

**FIG. 3. NO donor blocks intersubunit cross-links induced by diamide.** A, Coomassie Blue stained 5% SDS non-reducing polyacrylamide gel showing the effect of 100 μM SNAP on diamide cross-linking of RYR1 subunits. Lane 1 shows 20–25 μg of SR under non-reducing condition (a, 565 kDa RYR1; b, 400 kDa). Lane 2 shows SR membranes treated with 500 μM diamide for 10 min at room temperature (A, 2 × 565 kDa; B, 2 × 400 kDa). Lane 3 shows SR membranes treated with 100 μM SNAP prior to treatment with 500 μM diamide (*std*, molecular weight standard). B, this figure shows the effect of NO donors on diamide-induced cross-links. Data shown as mean S.E. (*n* = 9). The y axis shows the percentage of cross-linking in the presence of NO donors relative to cross-linking in the absence of NO donors. NO donors significantly decrease cross-linking by diamide (*p* < 0.01; one-tailed t test).

![Figure 4](http://www.jbc.org/)

**FIG. 4. Lower concentrations of SNAP have no effect on RYR1 reconstituted into planar lipid bilayer.** A, single channel traces of RYR1 reconstituted into planar lipid bilayer. First trace (top) shows control records of RYR1 (O and C reflect the open- and closed-state of the channel, respectively). The effect of 100 μM SNAP added to the *cis* chamber is seen in the second trace. Addition of 500 μM diamide to the *cis* chamber (after 100 μM SNAP) is seen in the third trace. Fourth trace (bottom) represents records when 5 mM NEM was added to the *cis* chamber (after 100 μM SNAP and 500 μM diamide). B, histogram showing open time as a fraction of total time (*P*, measured in 1-min bins) during sequential addition of 100 μM SNAP, 500 μM diamide, and 5 mM NEM.
NO Interaction with the Ca\(^{2+}\) Release Channel

**Fig. 5.** Higher concentrations of NO donors increase RYR1 activity and induce intersubunit cross-links. A, single channel traces of RYR1 reconstituted into planar lipid bilayer and exposed to either NOC-15 (left traces) or NOC-9 (right traces). Addition of 500 \(\mu\)M NOC-15 or 5 \(\mu\)M NOC-9 to the cis chamber has no effect on single channel activity; subsequent increases to 5 mM NOC-15 or 0.5 mM NOC-9 caused channel activity to increase 10–15-fold. The results are summarized in Table II. B, this plot was obtained using the optical densities of 565 kDa RYR1 monomer measured on Coomassie stained 5% SDS-polyacrylamide gel electrophoresis after incubation with NOC-15, 0.05–25 mM (●) or NOC-15 followed by 200 \(\mu\)M diamide (○). NOC-15 concentrations between 0.1–1 mM prevent disappearance of monomer, whereas monomer is progressively lost at NOC-15 concentrations greater than 2.5 mM.

**Table II**

<table>
<thead>
<tr>
<th>Effect of NO donors on single channel activity</th>
<th>SNAP(^{a})</th>
<th>NOC-15(^{b})</th>
<th>NOC-9(^{c})</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>P&lt;sub&gt;o&lt;/sub&gt; = 0.012 ± 0.003 (n = 10)</td>
<td>0.020 ± 0.007 (n = 4)</td>
<td>0.014 ± 0.005 (n = 6)</td>
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<tr>
<td>Low concentration</td>
<td>P&lt;sub&gt;o&lt;/sub&gt; = 0.013 ± 0.004 (n = 4)</td>
<td>0.019 ± 0.008 (n = 3)</td>
<td>0.020 ± 0.004 (n = 4)</td>
</tr>
<tr>
<td>High concentration</td>
<td>P&lt;sub&gt;o&lt;/sub&gt; = 0.106 ± 0.105(^d) (n = 10)</td>
<td>0.215 ± 0.041(^e) (n = 4)</td>
<td>0.212 ± 0.064(^e) (n = 6)</td>
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</table>

\(^{a}\) Half-life = 40–60 min; 100 \(\mu\)M produces a peak value for free in the nanomolar range. Low concentration, 0.1 mM; high concentration, 1 mM.  
\(^{b}\) NOC-15, PAPA NONOate, half-life = 76 min; 0.5 mM produces a peak value for free NO in the nanomolar range. Low concentration, 0.2–0.5 mM; high concentration, 5 mM.  
\(^{c}\) NOC-9, MAMA NONOate, half-life = 2.5 min; 500 \(\mu\)M of donor produces a peak value for free NO in the micromolar range. Low concentration, 0.005 mM; high concentration, 0.5 mM.  
\(^{d}\) In three out of ten experiments, there was an increase in P<sub>o</sub>; higher concentrations of SNAP in our system are not feasible.  
\(^{e}\) P < 0.01, Student’s paired t test with Bonferroni correction was used to determine the significance of change.

ments, SNAP had no significant effect on the activity of the channel over a 30-min incubation period. The P<sub>o</sub> was 0.012 ± 0.003 under control conditions, 0.013 ± 0.004 (n = 4) after 10 min, and 0.010 ± 0.005 (n = 3) after 30 min in the presence of SNAP. Subsequent diamide treatment did not activate the channel (P<sub>o</sub> = 0.011 ± 0.004, (n = 3)). Phase 2 NEM alkylation can, however, further activate diadime-treated channels (1). After treatment with SNAP, NEM was still able to increase channel activity (P<sub>o</sub> = 0.162 ± 0.0314 (n = 3, p < 0.05)), indicating that phase 2 sulphydryls had not reacted with diamide or SNAP.

**Higher Concentrations of NO Donors Activate RYR1 Reconstituted Into Planar Lipid Bilayer**—Stoyanovsky et al. (10, 11) reported an increase in single channel activity with 0.5–1 mM SNAP at \(p\)Ca of 7. To determine if NO donors have a biphasic effect on RYR1 activity we used higher concentrations of NO donors on the reconstituted channel in planar lipid bilayer. Fig. 5A shows the effect of high and low concentrations of NOC-9 and NOC-15 on single channel activity. Low concentrations of NO donors, 500 \(\mu\)M NOC-15 and 5 \(\mu\)M NOC-9, had no effect on single channel activity whereas high concentrations, 5 mM NOC-15 and 0.5 mM NOC-9, increased activity. Table II summarizes the results obtained using NOC-9, NOC-15, and SNAP. We subsequently tested the effect of NO concentration on dimer formation. Fig. 5B shows that NOC-15 concentrations higher than 2.5 mM induce cross-links between RYR1 subunits. However if NOC-15 treated membranes are subsequently treated with 200 \(\mu\)M diamide, a biphasic effect of NOC-15 is seen. At concentrations between 0.1–1 mM, NOC-15 prevents cross-linking by diamide as shown by an increase in the amount of monomer. At higher concentrations (>2.5 mM), monomer levels decrease indicating cross-linking by NOC-15.

**DISCUSSION**

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a physiological oxidant that has been shown to increase intracellular Ca\(^{2+}\) and enhance contractile function of skeletal muscle fibers in the resting state (3, 20). Endogenous NO is produced at 1–3 pmol/mg by skeletal muscle fibers and has been shown to depress contractile function (4, 5). In unfatigued muscle, therefore, NO acts to oppose the effects of ROI on contractile function, perhaps by preventing increases in intracellular calcium. Strenuous contractile activity accelerates the production of both ROI and NO, leading to the accumulation of oxidants and loss of contractile function.
during muscle fatigue. High concentrations of H$_2$O$_2$ and other ROI, however, cause oxidative damage and may contribute to eventual cell death.

We have shown that diamide produces intersubunit cross-links within the RYR1 tetramer and enhances channel activity (1, 18). Present data demonstrate that hydrogen peroxide induces intersubunit cross-links and activates the RYR1 reconstituted into planar lipid bilayer. Similar to diamide, phase 1 alkylation by NEM is blocked by H$_2$O$_2$. These observations suggest a common mechanism for the effect of oxidants on RYR1, i.e. oxidants produce disulfide bonds between neighboring subunits on RYR1 and, in doing so, increase channel activity. This ability of oxidants to increase the activity of RYR1 could explain the effects of oxidants on contractile force production by skeletal muscle. In contrast to oxidants, NO decreases contractile force generation in unfatigued skeletal muscle (4). Mészáros et al. (8, 9) reported an inhibitory effect of NO on RYR1 activity which would explain the decrease in contractile function. In our experiments, NO donors at lower concentrations did not change channel activity but did inhibit intersubunit cross-linking and prevent the activation of RYR1 by oxidants.

Previous work in other laboratories show activation of RYR1 by NO donors (10, 11). These findings are consistent with our work using higher donor concentrations (Table II). The higher concentration of NO donors required for activation in our studies may reflect the buffer conditions used. Neither ATP nor caffeine were in our assay. NOC-15 has a slow rate of NO release (25) and requires millimolar concentrations to activate the channel. NOC-9 has a shorter half-life and activates the channel at sub-millimolar concentrations. The activation of RYR in bilayer is consistent with intersubunit cross-linking at higher concentrations of NOC-15 and NOC-9. SNAP at 1 mM activated the channel only 3 out of 10 times and did not induce intersubunit cross-links. We attribute the ineffectiveness of SNAP to its low solubility in our buffer system or that it might be producing different NO species than NONOates. Regardless of the difference seen at higher donor concentrations, we find: 1) all NO donors blocked phase 1 of NEM alkylation, whereas their by-products after releasing NO did not; 2) all donors decreased cross-linking by diamide at subactivating concentrations of donors. Our findings suggest a possible mechanism for the opposing effects of oxidants and NO on force generation in skeletal muscle; oxidants activate the channel by forming intersubunit cross-links, and at lower concentrations NO prevents this activation and intersubunit cross-linking. However, higher NO concentrations contribute to oxidative pressure and promote activation of the RYR1. The two effects of NO probably result from interaction with distinct sulfhydryls. These interactions of NO with RYR1 may play an important role in modulating excitation-contraction coupling and muscle fatigue.

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