Published in final edited form as:

Free Radic Biol Med. 2010 December 15; 49(12): 1969–1977. doi:10.1016/j.freeradbiomed.2010.09.025.

REDOX MODULATION OF DIAPHRAGM CONTRACTILITY: INTERACTION BETWEEN DHPR and RyR CHANNELS

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Abstract

Previous reports indicate that reactive oxygen species (ROS) may modulate contractility in skeletal muscle. Although Ca^{2+} -sensitivity of the contractile apparatus appears to be a primary site of regulation, dihydropyridine receptor (DHPR or L-type Ca^{2+} channels) and calcium efflux in isolated sarcoplasmic reticulum (SR) vesicles appear to be redox sensitive as well. However, DHPR as a target is poorly understood in intact muscles at body temperature, particularly in the diaphragm, a muscle more dependent on external Ca^{2+} than locomotor muscles. Previously, we reported that oxidant challenge via xanthine oxidase (XO) alters the K^{+} contractures in diaphragm fiber bundles, suggestive of a role of L-type Ca^{2+} channels. Contractility of isolated rat diaphragm fiber bundles revealed a biphasic response to ROS challenge that was dose and time dependent. Potentiation of twitch and low-frequency diaphragm fiber bundle contractility with $0.02 \text{ U} \cdot \text{ml}^{-1}$ XO was reversible or partially preventable with washout, dithiothreitol, and the SOD/catalase mimetic EUK-134. The RyR antagonist ruthenium red inhibited xanthine oxidase-induced potentiation, while the RyR agonist caffeine elevated diaphragm twitch and low-frequency tension in a non-additive manner by 55% when introduced simultaneously with ROS challenge. The DHPR antagonist nitrendipine ($15 \mu\text{M}$) inhibited elevation in low-frequency diaphragm tension produced by ROS challenge. Caffeine threshold tension curves were shifted to the left with $0.02 \text{ U} \cdot \text{ml}^{-1}$ XO, but this effect was partially reversed with $15 \mu\text{M}$ nitrendipine. These results are consistent with the hypothesis that DHPR redox state and RyR function are modulated in an interactive manner, affecting contractility in intact diaphragm fiber bundles.

Keywords

skeletal muscle; reactive oxygen species; xanthine oxidase; DHPRs; L-type Ca^{2+} channels

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INTRODUCTION

Previous studies indicate that reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) modulate contractile function in skeletal muscle. In an unfatigued state, tension development in the isolated diaphragm muscle fibers decreased with the application of antioxidants such as N-acetylcysteine, superoxide dismutase, and catalase [1,2,3]. Modulation of skeletal muscle fiber tension by hydrogen peroxide [4] and nitric oxide [5] has been previously reported. We also reported that the ROS donor xanthine oxidase increased twitch and low-frequency contractility of the unfatigued diaphragm [1]. In contrast, loss of tension with fatiguing stimulation may be attenuated to a modest degree by antioxidants, or even exacerbated depending on the nature of the antioxidant [1,2,6]. Contractile state, glutathione status, type of ROS, muscle, temperature, and age can play important roles in the responsiveness of skeletal muscle contractility to changes in the redox environment [7].

Reactive oxygen species appear to target multiple sites including the contractile apparatus and possibly Ca^{2+} channels [8,9,10]. Alterations in Ca^{2+} sensitivity of the contractile apparatus consistently contribute to ROS-induced augmentation of force in quiescent muscle and contribute to muscle fatigue [8,11]. ROS may also target dihydropyridine-sensitive, voltage sensing L-type Ca^{2+} channels (DHPRs), located in the t-tubules, and the ryanodine (RyR)-sensitive Ca^{2+} release channels of the sarcoplasmic reticulum (SR) [9]. For example, the ability of ROS to alter the dynamics of muscle K^{+} contractures is believed to be dependent of DHPRs and their influence on RyRs [9,12]. In addition, oxidants also altered the release of Ca^{2+} from both isolated sarcoplasmic reticulum vesicles and single-channel ryanodine-sensitive Ca^{2+} channels (RyRs) [13,14]. Integration of cellular redox state into mechanisms that govern Ca^{2+} efflux from isolated sarcoplasmic reticulum was illustrated by Favero *et al.* [15], who found that hydrogen peroxide altered the sensitivity of rat muscle SR vesicles to ligands such as Ca^{2+} , Mg^{2+} , ATP, and $[\text{H}^{+}]$.

In mechanically skinned rodent and toad fibers, ROS modulation of DHPR function and SR Ca^{2+} release is uncertain [14,16]. Reports using single fiber experiments cited ROS modulation of Ca^{2+} sensitivity of the contractile apparatus as the primary effect [8,17,18]. However, it is possible that room temperature used in many single fiber preparations may alter cell membrane fluidity, cholesterol state, and therefore redox sensitivity of membrane-bound proteins [19,20]. This may be particularly true at temperatures below 32°C [20].

Oba *et al.* [4] and Salama *et al.* [10] suggested that ROS could impact function of dihydropyridine-sensitive L-type Ca^{2+} channels (DHPRs), and thus affect skeletal muscle contractility, in intact fibers or muscles. Aoki *et al.* [21] found that inward Ca^{2+} currents of frog single fibers in response to Ag^{+} were diminished by dithiothreitol (DTT). However, there is currently little data investigating redox modulation of DHPR function as a modulator of diaphragm contractility. Previously, we found oxidant challenge altered the size and shape of muscle K^{+} contractures [9]. K^{+} contracture time-to-peak tension and relaxation rate, hypothesized to be a function of DHPRs [9,12] were significantly altered. The influence of redox modulation of DHPR function of intact muscle is especially intriguing in the diaphragm, as this unique inspiratory muscle is more dependent on external Ca^{2+} than limb muscles [22,23]. Thus the intact diaphragm may express sensitive redox modulation of DHPR and RyRs like the heart [24].

Recent data indicate the importance of xanthine oxidase, located in the muscular vasculature, in stimulating nuclear factor-kappaB and cell signaling in exercising muscle [25]. Ji and colleagues have cited this phenomenon as an example of “hormesis” in responding to the stressors of activity [25]. Moreover, as antioxidant enzymes and substrates

are virtually non-existent in interstitial space, ROS and RNS are free to roam and thus participate in intercellular signaling, metabolism, and potentially contractile function. In addition, sarcolemmal NAD(P)H oxidase and other extramitochondrial sites may be more important ROS sources than mitochondria during unfatigued muscle contractions [26].

Therefore, we tested the hypotheses that exposure of diaphragm fiber bundles to extramyocyte ROS challenge will result in potentiation of low-frequency tension that is a) a reversible phenomenon with washout and preventable with reducing agents, and b) dependent on DHPR Ca^{2+} channels and their interaction with RyR Ca^{2+} channels.

METHODS

Animals

Four-month-old male Fischer-344 rats were housed and cared for in accordance with American Physiological Society policy. Animals were maintained on a 12hr light/12hr dark cycle, and rat chow and water provided *ad libitum*. The University Laboratory Animal Care Committee approved the animal use protocol (AUP) prior to the commencement of experiments.

in vitro preparation

Preparation of diaphragm fiber bundles was an adaptation of that previously described [27]. Animals were anesthetized using $50 \text{ mg}\cdot\text{ml}^{-1}$ Na pentobarbital, and the whole diaphragm was then quickly excised (<30 sec without blood flow). Diaphragms were immediately placed in ice-cold (4°C) Krebs solution aerated with a 95% O_2 , 5% CO_2 gas mixture. The Krebs solution ($\text{pH} = 7.40$) contained the following: 118mM NaCl, 4.7mM KCl, 1.8mM CaCl_2 , 1.18mM MgSO_4 , 1.18mM KH_2PO_4 , 25mM NaHCO_3 , and 11 mM glucose. Two small fiber bundles were then carefully removed from the lateral portion of the costal diaphragm with the rib intact on one end, and the central tendon intact on the opposite end. We used silk 4-0 sutures to tie the rib end of each diaphragm fiber bundle to a glass rod holder, with another suture tied to the central tendon. Each fiber bundle was placed in a muscle bath (Harvard Apparatus) containing Krebs solution at 36.5°C ($\text{pH}=7.40$; bubbled with 95% O_2 , 5% CO_2), with the central tendon suture secured to an isometric force transducer (Grass FT03). Tension was processed by a DC amplifier, displayed on a chart recorder (Allen Datagraph 2125E), and data stored on a Mac computer. Following a 10-minute equilibration period, we determined the optimal length (L_0) to achieve peak twitch tension. All subsequent measurements were then made at L_0 . Fiber bundles were stimulated by field generation through two platinum foil electrodes driven by a Grass stimulator (S48) in series with a current regulator. Stimulus strength was set at 1.5 times the voltage needed to produce peak twitch tension. Stimulus trains were 500 ms in duration and each pulse within a train had a length = 2 msec. Twitch, 20 Hz (low-frequency), and 120 Hz (maximal isometric tension: P_0) were used as a pulse paradigm to assess the contractile function of diaphragm fiber bundles [27]. Twitch time-to-peak tension and one-half relaxation time ($1/2 \text{ RT}$) were assessed using an HP digital oscilloscope. One minute was allowed between each stimulus to eliminate hysteresis. Mean P_0 levels were $27.8 \pm 0.6 \text{ N}\cdot\text{cm}^{-2}$ for baseline diaphragm fiber bundle measurements.

Xanthine oxidase was used as a physiological ROS donor. Xanthine oxidase is found in substantial concentrations associated with skeletal muscle vascular endothelium in both humans and animals [28,29]. Even though much of xanthine oxidase is in the dehydrogenase form, it can be converted to the oxidase form readily during exercise and ischemia-reperfusion due to cleaving by Ca^{2+} or ROS [25,30]. Thus XO served as a physiological ROS (primarily superoxide) generator [31] and as a model for endogenous NAD(P)H

oxidase and xanthine oxidase as well since hydrogen peroxide diffuse easily and superoxide anions translocate via anion channels across plasma membranes (Fisher 2009). Both hydrogen peroxide and superoxide anions have a half-life long enough to allow some movement from the site of production [9]. Data from Ji and colleagues [25] indicate the importance of xanthine oxidase in muscle cell signaling and stress protein activation. In our experiments protease-free xanthine oxidase was purchased from Biozyme and Sigma.

Experimental Design

Dose and Time Effect of XO—The purpose of this experiment was to determine if potentiation of unfatigued diaphragm contractility by xanthine oxidase was dose-dependent. The content of the muscle bath medium served as the treatment. Two diaphragm fiber bundles, from the left and right lateral costal portions of the whole diaphragm, were taken from each animal, with each diaphragm served as its own control. Following preparation of diaphragm fiber bundles as described above, baseline contractile measures were taken (twitch, 20 Hz, and 120 Hz).

Fiber bundles were assigned to one of the following treatment groups (n=8/group) by content of the bath media: 0.01 U•ml⁻¹ xanthine oxidase + 200 μM hypoxanthine in Krebs solution; 0.02 U•ml⁻¹ xanthine oxidase + 400 μM hypoxanthine; 0.03 U•ml⁻¹ xanthine oxidase + 600 μM hypoxanthine; 0.05 U•ml⁻¹ xanthine oxidase + 1 mM hypoxanthine; and controls consisting of 1 mM hypoxanthine in Krebs solution (CON). We have verified production of superoxide anion in the bath medium by XO previously using cytochrome c reduction determined spectrophotometrically as described [1]. Following the introduction of our test solutions, 5 minutes was allowed for equilibration and diaphragm contractile properties were re-measured at 5 (POST-5) and 10 minutes (POST-10). Following another 60 minutes in the test solutions, another set of contractile measures for diaphragm fiber bundles were taken (POST-70) to determine the prolonged effect of exposure to oxidative stress.

Prevention or Reversibility of XO Potentiation of Contractility—We tested the hypothesis that xanthine-induced potentiation of diaphragm contractility with xanthine oxidase was a phenomenon (a) reversible by washout with Krebs solution without xanthine oxidase (0.02 U•ml⁻¹) or hypoxanthine or (b) preventable via use of inactivated xanthine oxidase via a UV source or the use of reducing or antioxidant agents. The reducing agent dithiothreitol and the superoxide dismutase/catalase mimetic EUK-134 (50 μM) were used to determine if XO-induced (0.02 U•ml⁻¹) contractile changes were a function of oxidative stress. Diaphragm fiber bundles (n=8 rats per group) were assigned to the following groups: Krebs + 400 μM hypoxanthine (CON); Krebs + 0.02 U•ml⁻¹ xanthine oxidase + 400 μM hypoxanthine (XO); Krebs + 400 μM hypoxanthine + inactive xanthine oxidase protein (Inactive XO); Krebs + xanthine oxidase + hypoxanthine + 2 mM dithiothreitol (XO + DTT); xanthine oxidase + hypoxanthine + 30 μM EUK-134. The activity of irradiated xanthine oxidase assessed via cytochrome c reduction measured at 500 nm (Lawler *et al.* 1997) and determined to be less than 5% of native enzyme. Pre- and post-treatment contractile measures were performed on each diaphragm fiber bundle. We found no differences between 5 and 10 min readings, and used 5 min time points for these and following experiments.

Measurement of Oxidative Stress—To verify that ROS levels were elevated inside diaphragm muscle cells, we targeted dichlorohydrofluorescein diacetate (DCFH: Molecular Probes) oxidation as modified from a technique outlined previously [32] for confocal microscopy. We isolated diaphragm fiber bundles (n=8 rats/group) and divided into loaded the muscle with DCFH (50 μM) in Krebs with 400 μM hypoxanthine (CON), 0.02 U•ml⁻¹

XO + 400 μ M hypoxanthine (XO), XO + hypoxanthine + 1 mM DTT, and XO + hypoxanthine + 15 μ M nitrendipine. Nitrendipine was tested to determine whether concentrations we had titrated for DHPR-blocking experiments would reduced oxidation. Samples were then incubated on a shaker at 37°C for 10 min. We used 490 nm as our excitation and 530 as our emission detection wavelengths. Diaphragm fiber bundles were loaded for 20 minutes with DCFH. Fluorescence was monitored using a confocal microscope (Zeiss LSM410) via an interface with a PC computer. After baseline measures, DCFH oxidation was followed over 10 minutes via fluorescent emission intensity.

RyR Antagonism and XO-induced Potentiation—Here we tested the hypothesis that the RyR antagonist ruthenium red would inhibit increased in low-frequency tension induced by xanthine oxidase (0.02 U•ml⁻¹). Following baseline contractile measures, fiber bundles (n=8 rats per group) were assigned to the following groups: Krebs + 400 μ M hypoxanthine (CON); Krebs + 0.02 U•ml⁻¹ xanthine oxidase + 400 μ M hypoxanthine; xanthine oxidase + hypoxanthine + 30 μ M ruthenium red; and Krebs + 30 μ M ruthenium red.

RyR Agonists and XO-induced Potentiation—In this experiment we tested the hypothesis that the RyR agonist caffeine would enhance xanthine oxidase (0.02 U•ml⁻¹) induced increases in low-frequency diaphragm tension in a non-additive manner. Following baseline contractile measures, diaphragm fiber bundles (n=8 rats per group) were assigned to the following test groups: Krebs + 400 μ M hypoxanthine (CON); Krebs + 0.02 U•ml⁻¹ xanthine oxidase + 400 μ M hypoxanthine; Krebs + 1 mM caffeine; Krebs + XO + hypoxanthine + 1 mM caffeine. A parallel experiment was conducted with 5 μ M chloro-m-cresol instead of caffeine to verify the effects of XO on RyR function.

DHPR Antagonists and XO-induced Potentiation—We investigated the effect of the DHPR antagonist nitrendipine (15 μ M) on XO-induced potentiation of diaphragm contractility. Following baseline contractile properties measures, diaphragm fiber bundles (n=6 per group) were assigned to the following: Krebs + 400 μ M hypoxanthine (CON); Krebs + 0.02 U•ml⁻¹ xanthine oxidase + 400 μ M hypoxanthine; Krebs + 15 μ M nitrendipine, and xanthine oxidase + hypoxanthine + 15 μ M nitrendipine.

XO, DHPR Antagonism and the Caffeine Threshold—Here we tested the hypothesis that changes in redox state resulted in an interactive effect on DHPR and RyR Ca²⁺ channels. We predicted that XO would lower the “caffeine threshold” needed to elicit increased baseline tension in muscles. We postulated that the DHPR antagonist nitrendipine would partially reverse the XO-induced (0.02 U•ml⁻¹) left shift of the caffeine threshold curve. Fiber bundles (n=8 for each group) were exposed to one of the following test solutions: Krebs solution with 400 μ M hypoxanthine (CON); Krebs + 0.02 U•ml⁻¹ xanthine oxidase + hypoxanthine; Krebs + 30 μ M nitrendipine; and XO + hypoxanthine + 30 μ M nitrendipine. Tension was monitored continuously while boluses of caffeine stock were introduced into the tissue medium to raise caffeine concentration in 1 mM increments beginning with 0 mM to 14 mM, when a plateau was reached. Two minutes were allowed between each caffeine bolus. Maximal caffeine-elicited tension was verified in experiments (n=6) demonstrating no further increase in tension for 30 mM caffeine vs. 14 mM. We also found no evidence in pilot experiments that hysteresis was affecting caffeine-induced changes in tension.

Statistics

Two-way ANOVAs for repeated measures with Fishers-LSD used post-hoc where appropriate were used to assess changes in diaphragm tension to determine if mean

differences existed between the test groups for each of the 6 sets of experiments. Significance level was set at 0.05.

RESULTS

We found an incremental, significant elevation in twitch tension with diaphragm fiber bundles up to $0.02 \text{ U}\cdot\text{ml}^{-1}$ xanthine oxidase. However, potentiation of diaphragm twitch tension was attenuated at higher (i.e., 0.03 and $0.05 \text{ U}\cdot\text{ml}^{-1}$) XO concentrations (Figure 1). Low-frequency (20 Hz) diaphragm fiber bundle tension with 5 minutes of xanthine oxidase exposure displayed a similar “inverted U” relationship between tension and XO activity (Figure 1). In contrast, 5 minutes of exposure of diaphragm fiber bundles to XO did not significantly alter P_o at any XO concentration.

Prolonged exposure (70 min) of diaphragm fiber bundles to high levels of xanthine oxidase (0.03 and $0.05 \text{ U}\cdot\text{ml}^{-1}$) significantly depressed P_o and low-frequency contractility (Figure 2). Depression in both diaphragm low-frequency tension and P_o with prolonged exposure of diaphragm fiber bundles was associated with the highest concentration of XO ($0.05 \text{ U}\cdot\text{ml}^{-1}$) (Figure 2). In contrast, prolonged exposure of diaphragm fiber bundles to lower levels of XO (0.01 and $0.02 \text{ U}\cdot\text{ml}^{-1}$) did not depress diaphragm contractility. Parallel control experiments demonstrated that the preparation was viable over 70 min .

Five minutes of exposure of $0.02 \text{ U}\cdot\text{ml}^{-1}$ to xanthine oxidase induced potentiation of twitch and low-frequency tension by 34% and 37% , respectively (Figure 3). P_o of diaphragm fiber bundles was unaffected by xanthine oxidase exposure. In contrast, controls with hypoxanthine or hypoxanthine + inactivated xanthine oxidase did not display a significant change in twitch tension, tension at 20 Hz , nor P_o . The force-frequency curve was significantly shifted to the left by XO at lower frequencies (data not shown) with no differences at higher frequencies ($80, 100, 120 \text{ Hz}$).

Washout of xanthine oxidase also completely reversed the XO-induced potentiation of diaphragm contractility (Figure 3). Addition of the reducing agent dithiothreitol (DTT) resulted in a partial prevention of the elevation of diaphragm fiber bundle tension with XO. Again, P_o was not altered by xanthine oxidase ($0.02 \text{ U}\cdot\text{ml}^{-1}$), washout, or DTT. In addition, 50 U/ml of the SOD/catalase mimetic EUK-134 significantly attenuated upregulation of diaphragm fiber bundle contractility for twitch and 20Hz stimulation rates by ROS challenge (0.02 U/ml xanthine oxidase) (Figure 4). No alteration with contractility occurred with XO or XO + EUK-134. Furthermore, follow-up experiment demonstrated a small and non-significant trend (-11.4%) reduction in low frequency contractility occurred after 5 min of treatment with EUK-134 without xanthine oxidase (data not shown). XO did not alter twitch dynamics (TPT or $1/2 \text{ RT}$) (data not shown). The lack of change in the relaxation phase is notable as it is inconsistent with Ca^{2+} -ATPase inhibition.

Elevation of oxidative stress was confirmed by pre-loading diaphragm fiber bundles with DCFH for 10 min and adding Krebs, Krebs + 0.02 U/ml XO + $400 \mu\text{M}$ hypoxanthine, or XO + 1 mM dithiothreitol (DTT). DCFH oxidation increased to $160 \mu\text{M}$ H_2O_2 equivalents with ROS challenge, and was significantly attenuated with the reducing agent DTT (Figure 5). DTT partially prevented XO-induced elevation of DCFH oxidation. In contrast, the DHPR antagonist nitrendipine had no effect on DCFH oxidation with or without XO-induced ROS challenge.

The RyR Ca^{2+} channel inhibitor ruthenium red significantly inhibited potentiation in diaphragm tension caused by $0.02 \text{ U}\cdot\text{ml}^{-1}$ XO (Figure 6). Both twitch and low-frequency tension were not different than controls when XO and ruthenium red were combined. Note that $30 \mu\text{M}$ ruthenium red alone had no effect on diaphragm contractility. Indeed, statistical

analysis revealed a significant interaction between XO and ruthenium red. In contrast, higher concentrations of ruthenium red (100 μM) depressed both low and high frequency tension significantly (data not shown). We also observed that the RyR Ca^{2+} channel antagonist procaine attenuated XO-induced potentiation as well (Figure 7).

The RyR agonist caffeine (1 mM) resulted in a significant increase in diaphragm twitch and low-frequency tension (Figure 8). Caffeine also significantly augmented the potentiation of low-frequency and twitch tension elicited by $0.02 \text{ U}\cdot\text{ml}^{-1}$ XO in a non-additive manner. Indeed, a significant interaction between XO and caffeine was detected statistically. Caffeine + XO increased low frequency tension by 57% without affecting P_o . These data are consistent with the hypothesis that application of an extramycocyte ROS source (xanthine oxidase) affects tension via RyR Ca^{2+} channels or upstream regulators (e.g., DHPRs), located in close proximity with t-tubules and sarcolemma.

The DHPR antagonist nitrendipine (15 μM) significantly inhibited increases in diaphragm low-frequency (20 Hz) tension caused by $0.02 \text{ U}\cdot\text{ml}^{-1}$ xanthine oxidase (Figure 9). XO increased low-frequency tension by 44%. In contrast, XO + nitrendipine elicited only a 22% increase in diaphragm twitch tension, and a 12% elevation compared with controls, and significantly less than the XO group. However, 15 μM nitrendipine did not depress low-frequency diaphragm contractility on its own for twitch and low-frequency tetanic tension. There were no significant differences in P_o among controls, XO, nitrendipine, or XO + nitrendipine.

Increasing levels of caffeine caused a rise in diaphragm tension. The relationship between caffeine concentration and diaphragm tension followed a sigmoidal relationship (Figure 10). When diaphragm fiber bundles were exposed to xanthine oxidase prior to incremental caffeine levels, the relationship between caffeine concentration and diaphragm tension was significantly shifted to the left. In other words, at a given concentration of caffeine, higher diaphragm tension was measured. Xanthine oxidase had less impact on maximal caffeine contracture tension. Nitrendipine had no effect on the caffeine threshold or the shape of the caffeine concentration vs. diaphragm tension curve, when compared to controls. In fact, the curves were virtually identical. However, nitrendipine reversed approximately 50% of the leftward shift caused by XO to the caffeine concentration vs. diaphragm tension curve. Similar effects were found on the caffeine threshold by 20 μM of nifedipine, another DHPR antagonist (data not shown).

DISCUSSION

Our results reveal that potentiation of intact diaphragm fiber bundles with the physiological ROS donor xanthine oxidase is a reversible or preventable phenomenon by washout, antioxidant application, or a reducing agent. This phenomenon was indeed dose and duration dependent. Our study using mammalian fiber bundles with intact connective tissue demonstrates a substantial and functionally relevant increase in diaphragm tension that is dependent on DHPR function. Indeed, Ca^{2+} channels antagonists and agonists modulated potentiation of diaphragm fiber bundle tension by ROS in a non-additive manner, indicative a significant contribution of DHPRs and RyRs to ROS-induced potentiation. The importance of DHPR interaction with RyR function was demonstrated via attenuation of the left shift in the caffeine threshold by ROS challenge. A discussion of the physiological relevance of our findings follows.

Reversible and Biphasic Dose and Temporal Response to ROS Challenge

We demonstrated a dose and time dependent impact of the ROS donor xanthine oxidase on diaphragm contractility (Figures 1 & 2). Reid and colleagues [3] earlier suggested

previously an optimal redox state for proper skeletal muscle function. Indeed, high levels of hydrogen peroxide impair tension generation in rat diaphragm and frog muscle fibers while lower levels may enhance contractility [2,3,4]. A biphasic response to redox state has been indicated for both RyR Ca^{2+} channels [14] in the SR and Ca^{2+} sensitivity of the contractile apparatus [8,11]. Two distinct classes of sulfhydryl groups with different affinity for oxidants in RyRs were proposed [14]. Our data suggest that intact skeletal muscle may exhibit a similar biphasic contractile response to ROS as lower levels of xanthine oxidase markedly enhanced diaphragm contractility, while high levels ($0.05 \text{ U}\cdot\text{ml}^{-1}$) did not. In fact, the combination of high levels of XO and prolonged exposure resulted in a substantial loss in diaphragm contractility. Note that both high and low-frequency contractility were impaired with prolonged exposure to 0.03 and $0.05 \text{ U}\cdot\text{ml}^{-1}$ XO. This is suggestive of impaired Ca^{2+} sensitivity or oxidative damage to the contractile apparatus. Indeed, Andrade *et al.* [8] concluded that prolonged exposure of single mouse toe muscle fibers to H_2O_2 depressed force mainly due to reduced Ca^{2+} sensitivity. Moopanar & Allen [11] also demonstrated that a redox-dependent reduction in Ca^{2+} sensitivity occurred in fatigued isolated mouse flexors.

We also found that indeed an optimal level of xanthine oxidase ($0.02 \text{ U}\cdot\text{ml}^{-1}$) existed and produced the greatest potentiation ($>30\%$) on low-frequency contractility in diaphragm fiber bundles. Importantly, our data clearly demonstrate the reversible nature of ROS challenge in intact diaphragm fiber bundles by washout, and prevention of potentiation via the reducing agent dithiothreitol and the catalytic SOD/catalase mimetic EUK-134 (Figure 3,4). Moreover, inactivation of xanthine oxidase prevented potentiation of the diaphragm and ROS production. Together, these experiments verify the importance of redox modulation in altering diaphragm contractility.

Salama *et al.* [10] reported that chemically skinned frog fibers were extremely sensitive to redox changes with heavy metal exposure that elicited spontaneous twitches. We noticed no increase in baseline tension at $0.02 \text{ U}\cdot\text{ml}^{-1}$ XO in any experiment and only at the highest dose ($0.05 \text{ U}\cdot\text{ml}^{-1}$) with long-term exposure. Posterino and Lamb [18] reported that the oxidizing sulfhydryl agent DTDP ($100 \mu\text{M}$) did not initially affect baseline tension or tension caused by ion-induced depolarization in mechanically skinned single fibers from rats and toads at 23°C .

Extramyocyte ROS May Regulate DHPR Function and Diaphragm Contractility

Location of ROS exposure may be a contributing factor in affecting diaphragm contractility and DHPR and RyR Ca^{2+} channel function. Xanthine oxidase is primarily located in the muscle vasculature and NAD(P)H oxidase in the cell membrane [30,32]. Therefore, it is logical that ROS formed outside myocytes are in proximity to the sarcolemmal would be most likely to influence DHPR and RyR function. This is consistent with ROS compartmentalization theory discussed by Fisher (2009) [33]. Further, recent data also call into question the contribution of mitochondrial ROS release to muscle contractions [34]. Importantly, as contracting fibers release H_2O_2 and other ROS, and as the interstitial space is virtually devoid of antioxidant enzymes and scavengers, release of ROS via xanthine oxidase, NAD(P)H oxidase, and possibly subsarcolemmal mitochondria in surrounding contracting fibers and vasculature could indeed affect DHPR, RyR function and thus contractility. Thus it is possible that redox regulation of membrane proteins may affect muscle contractions *in vivo* [35]. Indeed, in a new publication, inhibition of xanthine oxidase with oxypurinol decreased force production in the soleus and EDL [36].

We previously reported [9] that ROS alter the dynamics of rat diaphragm K^+ contractures, dependent on DHPR function. Feng *et al.* [37] related redox modulation of RyRs to alterations in GSH/GSSG (reduced:oxidized glutathione levels). Plant *et al.* [38]

demonstrated that high levels of H₂O₂ (1 mM) increased the sensitivity of calcium-induced calcium release. Indeed, the regulatory roles of major thiol/disulfide complexes including reduced/oxidized glutathione, cysteine/cystine, and reduced/oxidized thioredoxin are now believed to be critical in eliciting ROS-induced of cell signaling and contractility in skeletal muscle [34]. In the current study, nitrendipine significantly inhibited XO-induced potentiation of low-frequency tension in diaphragm fiber bundles (Figure 6), consistent with the notion that ROS can impact DHPR function [39]. We found no significant direct antioxidant effect of nitrendipine (Figure 5) and thus its effects were likely on DHPRs. We also found similar inhibition by procaine, suggestive of a similar mechanism.

Previous studies using single fiber models have indicated Ca²⁺ sensitivity of the contractile apparatus as a primary target of redox regulation of muscle contractility [8,14,17,18]. While reasons for potential inconsistencies among studies regarding the contribution of redox modulation of DHPR function in intact muscle is unclear, *in vitro* preparation (skinned single fiber vs. chemically skinned vs. intact fiber bundle), muscles chosen (e.g., diaphragm vs. EDL, soleus, toe flexor), source and location of ROS production, and temperature (23-25°C vs. 36.5°C) may all play a role. The diaphragm is also more dependent on external Ca²⁺ than locomotor muscles [22], possibly a reflection of the significant presence of heart DHPR isoform and/or RyR3 isoform [40,41]. Heart DHPR and RyR3 isoforms, unlike the skeletal muscle DHPRs and RyR1s are highly correlated with calcium-induced calcium release. However, the functional role of RyR isoforms in skeletal muscle is uncertain, as the contractile force of the diaphragm in transgenic mice lacking the RyR3 gene was unchanged [42].

The effect of temperature on ROS production and redox sensitivity of membrane proteins is gathering support [19,20]. SR and t-tubule membrane fluidity and thus cholesterol state and membrane protein (i.e., DHPRs, RyRs) function could indeed be altered at cooler mammalian muscle temperatures and rendered less sensitive to redox state [19,20]. Intact fiber bundles and mechanically skinned single fibers may also have differential signaling between cytoskeletal proteins and the extracellular matrix (ECM) [43], which could be exacerbated by lower ambient temperature.

In addition, location of ROS source is also likely important in its gain on membrane-bound Ca²⁺ channels [33]. The importance of ROS at the membrane level or external to myocytes is increasingly recognized as a contributor to cell signaling and contractile function. Recently, xanthine oxidase, located in the microvasculature [25,30] was shown to affect locomotor muscle contractility [44]. In addition, inhibition of xanthine oxidase blunts exercise-induced upregulation of NF-kappaB, MnSOD, and eNOS [25,44]. Therefore, it is possible that extramyocyte ROS may affect DHPRs preferentially *in vivo*. Future studies should focus on this postulate.

In summary, our data revealed that the ROS donor xanthine oxidase elicited a potentiation of diaphragm contractility that was reversible, preventable, and dependent on oxidant dose and duration in intact fiber bundles at body temperature. Results using DHPR and RyR Ca²⁺ channel agonists and antagonists were consistent with a model predicting that the interactive role of DHPRs and RyRs are important in ROS-induced potentiation in contractility of the intact diaphragm. We specifically propose that (a) DHPR redox state plays an important direct or indirect role on the redox state and function of RyR Ca²⁺ channels in intact diaphragm muscle, and (b) extramyocyte and membrane-site sources of ROS can affect contractility in the diaphragm.

Acknowledgments

Funding for this study was provided by support from the American Lung Association, NIH (AR054084), and NSF (ANT 0649609). The authors would like to thank Scott Demaree for his technical assistance.

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Figure 1. Biphasic contractile acute response of diaphragm fiber bundles to xanthine oxidase (XO)

Dose-response contractile effect of with acute (10 min) exposure of diaphragm fiber bundles to XO at 0, 0.01, .0.01, 0.03, and 0.05 U/ml (in Krebs with 1 mM hypoxanthine). Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). There was a significant interaction between tension and XO concentration.

Figure 2. Dose-dependent depression of diaphragm fiber bundles to prolonged exposure to XO
Dose-response contractile effect of with prolonged (70 min) exposure of diaphragm fiber bundles to XO at 0, 0.01, .01, 0.03, and 0.05 U/ml (in Krebs with 1 mM hypoxanthine). Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). 0.03 and 0.05 U/ml XO resulted in a large loss of diaphragm fiber bundle tension.

Figure 3. Effects of acute (5 min) exposure of diaphragm fiber bundles to XO are reversible with washout, inactivation of XO, and the reducing agent dithiothreitol)
 Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). (*) 0.02 U/ml XO significantly increased twitch and low-frequency diaphragm fiber bundle tension. (a) represents significantly different in tension than the XO group.

Figure 4. Effects of acute (5 min) exposure of diaphragm fiber bundles to XO are prevented with 100 μ m EUK-134 (SOD/catalase mimetic)

Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). (*) 0.02 U/ml XO significantly increased twitch and low-frequency diaphragm fiber bundle tension. (a) represents significantly different in tension than XO group.

Figure 5. Acute (5 min) exposure of diaphragm fiber bundles to XO increases DCHF (dichlorofluorescein-diacetate) oxidation

Oxidation of DCFH is expressed in H₂O₂ equivalents. 1 mM of reducing agent dithiothreitol (DTT) blunted DCFH oxidation, while DHPR antagonist nitrendipine (15 μ M) had not effect. (*) indicates significantly higher than controls. (a) indicates significantly lower than 0.02 U/ml xanthine oxidase.

Figure 6. The RyR antagonist ruthenium red blocks potentiation of increased twitch and low-frequency diaphragm contractions with 0.02 U/ml XO

Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_0). Changes in the contractile response of diaphragm fiber bundles was determined with 5 min. of exposure to Control (Krebs + 400 μM hypoxanthine), 0.02 U/ml XO, 30 μM ruthenium red, and 0.02 U/ml XO + 30 μM ruthenium red. (*) 0.02 U/ml XO significantly increased twitch and low-frequency diaphragm fiber bundle tension.

Figure 7. The RyR antagonist procaine reduces potentiation of increased twitch and low-frequency diaphragm contractions with 0.02 U/ml XO

Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). Changes in the contractile response of diaphragm fiber bundles was determined with 5 min. of exposure to Control (Krebs + 400 μ M hypoxanthine), 0.02 U/ml XO, 100 μ M ruthenium red, and 0.02 U/ml XO + 100 μ M ruthenium red. (*) 0.02 U/ml XO significantly increased twitch and low-frequency diaphragm fiber bundle tension. (a) is significantly lower than XO group.

Figure 8. The RyR agonist caffeine augments potentiation of increased twitch and low-frequency diaphragm contractions with 0.02 U/ml XO in a non-additive fashion

Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). Changes in the contractile response of diaphragm fiber bundles was determined with 5 min. of exposure to Control (Krebs + 400 μ M hypoxanthine), 0.02 U/ml XO, 1 mM caffeine, and 0.02 U/ml XO + 1 mM caffeine. Two-way ANOVA revealed a significant interaction between XO and caffeine. (*) 0.02 U/ml XO significantly increased twitch and low-frequency diaphragm fiber bundle tension. (a) indicates significantly higher diaphragm tension than 0.02 U/ml XO alone.

Figure 9. The DHPR antagonist nitrendipine partially blocks potentiation of increased twitch and low-frequency diaphragm contractions with 0.02 U/ml XO

Contractile responses were determined for a) twitch tension, b) low frequency tension at 20 Hz, and c) maximal tetanic tension (P_o). Changes in the contractile response of diaphragm fiber bundles was determined with 5 min. of exposure to Control (Krebs + 400 μ M hypoxanthine), 0.02 U/ml XO, 15 μ M nitrendipine, and 0.02 U/ml XO + 15 μ M nitrendipine. (*) 0.02 U/ml XO significantly increased muscle tension. (a) indicates significantly higher than 15 μ M nitrendipine alone.

Figure 10. 5 μ M of the DHPR antagonist nitrendipine partially prevents left shift of the caffeine-force relationship with ROS challenge

Effect of 5 min of exposure to Control (Krebs + 400 μ M hypoxanthine), 0.02 U/ml XO, 5 μ M nitrendipine, and 0.02 U/ml XO + 5 μ M nitrendipine on the caffeine [] -tension relationship. (*) indicates significantly higher than controls. XO resulted in a significant left shift of the curve. This was partially prevented by 5 μ M nitrendipine.