Po2 cycling protects diaphragm function during reoxygenation via ROS, Akt, ERK, and mitochondrial channels

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Zuo L, Pannell BK, Re AT, Best TM, Wagner PD. Po2 cycling protects diaphragm function during reoxygenation via ROS, Akt, ERK, and mitochondrial channels. Am J Physiol Cell Physiol 309:C759–C766, 2015. First published September 30, 2015; doi:10.1152/ajpcell.00174.2015.—PO2 cycling, often referred to as intermittent hypoxia, involves exposing tissues to brief cycles of low oxygen environments immediately followed by hypoxic conditions. After experiencing long-term hypoxia, muscle can be damaged during the subsequent reintroduction of oxygen, which leads to muscle dysfunction via reperfusion injury. The protective effect and mechanism behind Po2 cycling in skeletal muscle during reoxygenation have yet to be fully elucidated. We hypothesize that Po2 cycling effectively increases muscle fatigue resistance through reactive oxygen species (ROS), protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and certain mitochondrial channels during reoxygenation. Using a dihydrofluorescein fluorescent probe, we detected the production of ROS in mouse diaphragmatic skeletal muscle in real time under confocal microscopy. Muscles treated with Po2 cycling displayed significantly attenuated ROS levels (n = 5; P < 0.001) as well as enhanced force generation compared with controls during reperfusion (n = 7; P < 0.05). We also used inhibitors for signaling molecules or membrane channels such as ROS, Akt, ERK, as well as chemical stimulators to close mitochondrial ATP-sensitive potassium channel (KATP) or open mitochondrial permeability transition pore (mPTP). All these blockers or stimulators abolished improved muscle function with Po2 cycling treatment. This current investigation has discovered a correlation between KATP and mPTP and the Po2 cycling pathway in diaphragmatic skeletal muscle. Thus we have identified a unique signaling pathway that may involve ROS, Akt, ERK, and mitochondrial channels responsible for Po2 cycling protection during reoxygenation conditions in the diaphragm.

Skeletal muscles, such as the diaphragm, can generate reactive oxygen species (ROS) under normal resting conditions (46, 57, 66). However, when muscle tissue is continuously stimulated, the fibers experience a hypoxic insult that results in excessive amounts of ROS leading to oxidative stress while compromising muscle function (47, 57, 61, 64). After experiencing hypoxic environments, further tissue damage can occur as oxygen is quickly delivered to the muscle via reoxygenation (30).

Oxygen levels in skeletal muscle are depleted in varying degrees during exercise (8). Upon cessation of exercise, the reintroduction of oxygen and subsequent mitochondrial activity can induce the generation of ROS (8). Reoxygenation has also been suggested as a source of ROS in connective tissues, including tendons, leading to oxidative injuries (32). It has been reported that reoxygenation after a frostbite injury can induce damage that may be mediated by free radical formation (34). In frostbite models, antioxidants provided a protective effect during vascular reflow after dermal ischemia implying the role of ROS in reoxygenation injury (34). Furthermore, a previous study focusing on striated muscle compared reperfused with non-reperfused tissue after ischemia, which determined that reperfusion associated reoxygenation induces further injury that is beyond the damage caused by ischemia (53). We have previously demonstrated that Po2 cycling enhances fatigue resistance in contracting skeletal muscle (49, 60). Po2 cycling likely triggers specific signaling molecules to increase fatigue resistance during reoxygenation. However, the exact signaling pathway that mediates this protective effect has yet to be fully elucidated. This paper aims to further identify and characterize the signaling molecules associated with the pathways that underlie nonpharmacological preconditioning treatments, such as Po2 cycling, designed to protect skeletal muscle during reoxygenation (35).

We investigated the hypothesis that Po2 cycling significantly increases muscle fatigue resistance through an intracellular signaling cascade that includes ROS, protein kinase B (Akt), extracellular signal regulated kinase (ERK), mitochondrial ATP-sensitive potassium channel (KATP), and mitochondrial permeability transition pore (mPTP) during reoxygenation. Limited research has been performed to investigate ROS generation during diaphragm reoxygenation. Thus this can be an important area of research, as the diaphragm plays a critical role in respiration (42).

MATERIALS AND METHODS

Our overall strategy employed multiple inhibitors for the ROS/Akt/ERK pathway that mediates the Po2 cycling signaling cascade during reoxygenation of skeletal muscle using mouse models.

Animals. Our use of animals strictly adhered to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all procedures involving animals were approved and completed in accordance and compliance with The Ohio State University Institutional Animal Care and Use Committee regulations and guidelines. Adult male C57BL/6 mice (weight of ~20–30 g; average age of ~5 mo) were anesthetized with a combination of ketamine (70 mg/kg) and xylazine (10 mg/kg) via an intraperitoneal injection.

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Within 5 min, the diaphragm was removed and dissected into muscle strips (length: ~1.0 cm; width: ~0.5 cm) and mounted horizontally in a contracting chamber filled with Ringer’s solution (in mM: 21 NaHCO3, 1.0 MgCl2, 1.2 NaH2PO4, 0.9 Na2SO4, 2.0 CaCl2, 5.9 KCl, 121 NaCl, and 11.5 glucose; room temperature; pH 7.4).

Muscle function. Muscle function was measured using a myograph (model 800MS; Danish Myo Technology, Aarhus, Denmark). Before experimentation, muscles were stretched to an optimal length (15–20 min), the muscle was electrically stimulated to produce continual contractions for 5 min. Afterward, the muscle was stretched to an optimal length (15–20 min), the muscle was electrically stimulated to produce continual contractions for 5 min. Throughout stimulation, muscle bath solutions were maintained at approximately human body temperature (37°C) in accordance with previous protocols (38). Following contraction evaluation under hypoxia, muscle strips were immersed in 95% O2-5% CO2 to simulate hypoxia for 20 min. Diazoxide, cyclosporin A, and the inhibitor (PD98059; 100 μM; Promega) (51); the KATP channel opener diazoxide (100 μM; Sigma) (17); carboxyatractyloside, an ATP/ADP translocase inhibitor to facilitate mPTP opening (50 μM; Sigma) (9); and cyclosporin A, a drug that binds to cyclophilin D to prevent mPTP opening (100 μM; Cell Signaling Technology) (22), at room temperature (21°C). Following chemical treatment and a wash, muscle strips were preconditioned using alternating 2-min periods of 95% N2-5% CO2 and 95% O2-5% CO2. Because of the thickness of the mouse diaphragm, there is a long diffusion gradient that must be overcome in order for oxygen to permeate into the core of the tissue (59, 60). Therefore, we utilized more acute low oxygen and hypoxic conditions in vitro than would be expected in vivo to obtain the necessary level of cellular hypoxia and reoxygenation for our studies. For every muscle strip, the PO2 cycling protocol was repeated five times. Muscles not treated with PO2 cycling (control) were instead equilibrated in a Ringer’s solution bubbled with 95% O2-5% CO2 for 20 min. Diazoxide, cyclosporin A, and the combination of the two were administered to attempt to explore the PO2 cycling mechanisms using activators as positive controls. Control muscles were treated identically either in the absence of PO2 cycling or with the exception of inhibitor additions.

After PO2 cycling or equilibration periods, muscles strips were exposed to 95% N2-5% CO2 to simulate hypoxia for 30-min. During the middle of the hypoxia duration (~15–20 min), the muscle was electrically stimulated to produce continual contractions for 5 min. Throughout stimulation, muscle bath solutions were maintained at approximate human body temperature (37°C) in accordance with previous protocols (38). Following contraction evaluation under hypoxia, muscle strips were immersed in 95% O2-5% CO2 to simulate reoxygenation for a 15-min period. Continuous electrical stimulation of the muscles was introduced in the middle of the reoxygenation period (~5 min-10 min) to measure contractile force for 5 min.

Confocal microscopy. Confocal microscopy was performed to observe real-time ROS generation in both PO2 cycling-treated and control myofibers invisible to other methods of analysis. All imaging experiments were completed in a dark room utilizing a laser scan confocal microscope (Nikon confocal microscope D-Eclipse C1 system) to measure fluorescent signals. Superfused diaphragm strips were loaded into glass-bottomed culture dishes (MatTek, Ashland, MA) and perfused with a dihydrofluorescein diacetate (Hfluor) solution (40 μM; stock in dimethyl sulfoxide; Sigma) for 30 min. Once diffused into intracellular components, Hfluor reacts chemically and exhibits fluorescence in the presence of ROS (59). Hfluor also demonstrates less photobleaching and nitric oxide sensitivity than comparable probes such as dichlorofluorescein, which makes it ideal for our purposes (25, 59). Muscle strips were then secured in Ringer’s solution and sealed in the chamber to prevent fluctuations in oxygen levels or temperature. The muscle chambers were maintained at a normal physiological state (37°C) throughout the procedure. Tubing inlets were inserted to sustain hypoxic or hyperoxic muscle environments. The PO2 cycling protocol described earlier was performed on the microscope stage prior to baseline measurements for respective muscle strips. Ebselen (Alexis Biochemicals) was used as a ROS scavenger as described previously (59, 60, 65). Muscle strips were incubated in a 30 μM solution of ebselen simultaneously with the Hfluor probe. Control muscles did not receive either treatment.

Images (512 × 512 pixels) were recorded at 5-min intervals during a 10-min baseline, 30-min hypoxia treatment (95% N2-5% CO2), and 10-min reoxygenation treatment (95% O2-5% CO3) with setup parameters of laser: argon; pinhole: medium or large; excitation: 488 nm; and emission: 535 ± 25 nm. Background fluorescence was minimized as much as possible to diminish interference with ROS images throughout the experiment. Each image was analyzed using Adobe Photoshop 6.0, and the mean fluorescence was calculated for each image to determine intramuscular production of ROS.

Statistics. Data were analyzed for statistical significance utilizing one-way ANOVA, and sequentially expressed as means ± SE (IBM SPSS Statistics 21). The differences between treatments were identified via a series of post-ANOVA contrast analyses from IBM SPSS software. P < 0.05 was the determining criterion for paired (for comparing samples within the same group) or independent sample t-tests (for comparing samples between different groups).

RESULTS

Hfluor fluorescent diaphragm strips displaying representative images showing ROS levels are presented in Fig. 1. Images in Fig. 1, A, C, and E, taken via confocal microscopy before hypoxic exposure, demonstrate average resting levels of ROS. In contrast, images in Fig. 1, B, D, and F, represent fluorescence 5 min into the reoxygenation period after 30-min hypoxic periods. Our analysis revealed substantially increased mean fluorescent intensity during reoxygenation conditions in the control muscle strips (Figs. 1, A and B, and 2). This enhancement of ROS levels was completely abolished in muscles preconditioned with PO2 cycling (Fig. 1, E and F). These results were replicated when control muscles were incubated with ebselen, an antioxidant, confirming ROS as the determinant for fluorescence signaling (Fig. 1, C and D).

Grouped data summarizing all normalized fluorescence imaging during reoxygenation are illustrated in Fig. 2. Both PO2 cycling (n = 5) and ebselen (n = 5) trials yielded fluorescence significantly lower than control levels (n = 5) throughout the entire 10 min reoxygenation period (P < 0.001). This indicated that ROS generation increased dramatically throughout reoxygenation in controls, while PO2 cycling and ebselen-treated muscles retained low ROS levels during reoxygenation intervals.

As shown in Fig. 3, sample data depicting individual reoxygenation curves are represented by percentage of maximal contractile force after incubation in respective inhibitors. The control muscle contraction plot (Fig. 3A) serves a baseline analysis for diaphragmatic muscle. PO2 cycling treatment considerably improved muscle contractile force throughout reoxygenation, increasing both initial and final percentages (Fig. 3B). The effects of PO2 cycling on mitigating muscle fatigue

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were abolished after incubation with 1) Tiron and NAC in Fig. 3C; 2) nonmitochondrial inhibitors (ERK inhibitor, PD98059; AKT inhibitor, MK-2206) in Fig. 3, D and E; and 3) mitochondrial inhibitors (glibenclamide; carboxyatractyloside; glibenclamide + carboxyatractyloside; diazoxide + carboxyatractyloside) in Fig. 3, F - I. Contraction force levels comparable to the PO2 cycling curve were not observed in muscle strips incubated in cyclosporin A or diazoxide individually (Fig. 3, J and K, respectively). However, significant fatigue resistance was achieved through combinatorial incubation in diazoxide and cyclosporin A (Fig. 3L).

Grouped data confirmed significantly enhanced initial and final percentage of maximal contractions for PO2 cycling (Fig. 4; n = 7; P < 0.05). Figure 4 illustrates contractile strength for muscle strips incubated with antioxidants and nonmitochondrial inhibitors. Alternatively, Fig. 5 displays the data taken from muscle strips treated with mitochondrial activators or inhibitors. Only simultaneous exposure to diazoxide and cyclosporin A produced similarly significant muscle function data (Fig. 5; n = 8; P < 0.05) compared with PO2 cycling. However, it should be noted that individual incubation with cyclosporin A or diazoxide did significantly increase the initial percentage of contractile force compared with control (n = 5 and n = 7, respectively; P < 0.05). All other incubation preconditions diminished or eliminated fatigue resistance effects of PO2 cycling or did not result in grouped data significantly different from control (Figs. 4 and 5; n = 4 - 10; P < 0.05).

**DISCUSSION**

The results of our current study provide evidence indicating that the PO2 cycling protocols which we utilized in diaphragmatic skeletal muscle can diminish intracellular ROS levels during reoxygenation. The percentage of maximal contractile force of muscle tissues under reoxygenation conditions was significantly increased after treatment with PO2 cycling in vitro. However, addition of ROS, Akt, and ERK inhibitors negated PO2 cycling’s enhancement of muscle fatigue resistance. Interestingly, we were able to reproduce the increased percentage of maximal force in muscle strips without PO2 cycling through stimulation of mitochondrial KATP channel opening and mPTP channel closure. Collectively, these data demonstrate a correlation between reduced intracellular ROS levels and tissue protection in skeletal muscle during ischemia-reperfusion (I/R). Additionally, our data suggest that ROS, Akt, ERK, mitochondrial KATP channels, and mPTP channels all play important roles in the protective mechanism.

**Skeletal muscle.** It is important to note that there are some similarities between our data and published literature on ischemic preconditioning (IPC) in the heart. However, despite the physiological similarities between cardiac and skeletal muscle, our PO2 cycling method in skeletal muscle likely relies on a different mechanism not seen in IPC for cardiac muscle. Cardiac preconditioning protects heart function by preventing the trigger of necrotic cell death during ischemic-reperfusion injury. Alternatively, we propose that PO2 cycling triggers intracellular mechanisms that reduce skeletal muscle fatigue, not death, during reoxygenation. Muscle fatigue has been shown to be correlated with events such as increased ROS production (44), reduced myofibrillar Ca2+ sensitivity (39), and ATP depletion (2).
effect in tissues in vivo (52). Thus it is possible following PO2 cycling that increased expression of HIF-1α, via stimulated Akt and ERK, induces further activation of downstream genes and protects skeletal muscle during reoxygenation. Furthermore, Akt and ERK activation is already known to confer beneficial effects on skeletal muscle such as adaptation to exercise, increased ATP production, and mitochondrial biogenesis (16, 24). Therefore, Akt and ERK may also play a role in preconditioning skeletal muscle against fatigue. Interestingly, increased muscle fatigue resistance was also negated with the addition of the antioxidants Tiron and NAC, suggesting ROS participate in the protection mechanism (Figs. 3 and 4). Consequently, we have proposed a potential mechanism involving ROS signaling, PI3K, Akt, and ERK, which elucidates a possible pathway of the protective PO2 cycling effect in accordance with our data (Fig. 6).

**ROS mechanisms.** It has been previously confirmed that skeletal muscle produces ROS employing both intra- and extracellular sources, which generate higher concentrations as the muscle becomes overworked and fatigued (15, 47, 62). Recent research has also demonstrated ROS levels increase during sudden influxes of oxygen, such as during reoxygenation injury, through mitochondrial complexes and xanthine oxidase (XO) (40, 43). Under respiratory stress, intracellular xanthine dehydrogenase is converted to XO to produce ROS (6, 40). In addition, under normal conditions, mitochondrial complexes I and III can generate low amounts of ROS, serving as potential signaling molecules (26, 45). However, the exposure of a hypoxic environment to a sudden influx of oxygen causes the mitochondrial electron transport chain to experience excessive oxidant generation (56). This overproduction results in increased ROS accumulation, thereby overwhelming the cell’s endogenous antioxidant defense systems, dam-

**Signaling pathway.** We have previously demonstrated that inhibition of the phosphoinositide 3-kinase (PI3K) signaling pathway results in decreased potency of the preconditioning treatment in skeletal muscle (49). Our current study reports similar reduced effectiveness of PO2 cycling when treated with either Akt or ERK inhibitors in skeletal muscle (Fig. 4). These results are consistent with previous studies that suggest Akt or ERK is essential to hypoxia protection mechanisms. For example, overexpression of microRNA, miR-21, which can activate Akt and ERK, has been found to increase the prevalence of hypoxia-inducible factor-1α (HIF-1α) (31). In addition to Akt and ERK, HIF-1α has been determined to be an essential transcription protein for invoking the PO2 cycling protective

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**Fig. 3.** Sample reoxygenation curves displaying percentage of force compared with initial contraction incubated with numerous inhibitors. A: control; n = 10. B: PO2 cycling; n = 7. C: 1 mM Tiron (antioxidant) + 1 mM N-acetyl cysteine (NAC; antioxidant) + PO2 cycling; n = 6. D: 100 μM PD98059 (ERK inhibitor) + PO2 cycling; n = 5. E: 50 μM MK-2206 [protein kinase B (Akt) inhibitor] + PO2 cycling; n = 5. F: 100 μM glibenclamide [mitochondrial ATP-sensitive potassium channel (KATP) channel opening inhibitor] + PO2 cycling; n = 6. G: 50 μM carboxyatractyloside (ATP/ADP translocase inhibitor) + PO2 cycling; n = 5. H: 50 μM carboxyatractyloside (ATP/ADP translocase inhibitor) + 100 μM glibenclamide (KATP channel opening inhibitor) + PO2 cycling, n = 5. I: 100 μM diazoxide (KATP channel opener) + 50 μM carboxyatractyloside (ATP/ADP translocase inhibitor) + PO2 cycling, n = 5. J: 100 μM cyclosporin A (a drug that binds to cyclophilin D); n = 7. K: 100 μM diazoxide (KATP channel opener) + 100 μM cyclosporin A (a drug that binds to cyclophilin D); n = 8. Data represent approximate average contractile curve for each conditioning variable.

**Fig. 4.** Data illustrating the average percentage of maximal force at the first and end contractions during the 15-min reoxygenation period compared with initial strength immersed in nonmitochondrial channel inhibitors [control, n = 10; PO2 cycling, n = 7; 1 mM Tiron (antioxidant) + 1 mM NAC (antioxidant) + PO2 cycling, n = 6; 100 μM PD98059 (ERK inhibitor) + PO2 cycling, n = 5; and 50 μM MK-2206 (Akt inhibitor) + PO2 cycling, n = 5]. Baseline force measurements were calculated before exposure to hypoxic and reoxygenation conditions. *P < 0.05, significantly different from control.
PO2 cycling may trigger a preconditioning effect via the ROS/Akt/ERK pathway.

Data utilizing 3 or 10 PO2 cycling periods are not shown due to a lack of statistical significance, as such intervals did not optimize reoxygenation stress protection. This observation is likely due to the fact that 3 PO2 cycling periods are insufficient to develop muscle fatigue resistance and 10 PO2 cycling periods exert excessive hypoxic stress and subsequently harm the muscle. The current research proposes a preconditioning treatment that likely induces intracellular adaptations in reoxygenated skeletal muscle tissue. Thus our model has a similar effect to interval exercise, which also combats muscle fatigue or damage from oxidative stress presumably through enhanced antioxidant capacity (7, 14). Similarly intermittent hypoxic training, which involves patients using a mask to breath air with low oxygen concentrations, has been shown to increase tolerance to exercise (5, 27). As shown in Fig. 1, PO2 cycling attenuated ROS levels during reoxygenation in skeletal muscle. The abolishment of fluorescent signals after incubation with an antioxidant confirmed the substantial prevalence of ROS in controls. PO2 cycling treatment subsequently quenched the high levels of ROS (Fig. 2). It is plausible that the short hypoxic intervals of PO2 cycling increased signals that boost the intracellular antioxidant system and trigger the activation of scavengers, such as superoxide dismutase, to reduce excessive oxidant levels precipitated by reperfusion (10, 63).

Mitochondrial channels. Recent studies have shown that inhibition of the PI3K/Akt signaling pathway diminishes IPC-induced cardioprotection and PO2 cycling reoxygenation protection in skeletal muscle, highlighting the overall importance of this pathway to the preconditioning mechanisms (49, 55). The opening of mitochondrial KATP channels and closure of mPTP channels play critical roles in utilizing IPC to combat reperfusion injury (20, 22). Specifically, the opening of mitochondrial KATP channels causes an influx of cytosolic K+, activating K+/H+ antiports and contributing to matrix swelling (18, 54). It is plausible that the expanded matrix volume promotes cardioprotective effects by increasing ATP synthesis and fatty acid oxidation (17, 21). This correlation is paramount to our findings since cardiac and skeletal muscle tissues are both extremely redox sensitive and share similar structural characteristics. However, the endpoint of PO2 cycling in skeletal muscle is the reduced muscle fatigue while IPC mitigates necrotic cell death in cardiac muscle. Although the KATP and mPTP channels have been shown to play a role in cardioprotection, evidence has also shown these channels may play a protective role in skeletal muscle. It has been demonstrated that KATP deficiency increases resting tension leading to skeletal muscle fatigue in a mouse model (19). Furthermore, McAllister et al. (36) showed that the inhibition of mPTP opening in skeletal muscle is effective in protecting skeletal muscle.

Based on the current study, we propose that PO2 cycling promotes the opening of mitochondrial KATP channels and the closure of mPTP channels. Our data revealed that glibenclamide, a KATP closer, and carboxyatractyloside, an ATP/ADP translocase inhibitor, abolished the normal PO2 cycling-induced increase in percentage of contraction force during reperfusion (Figs. 3 and 5). Individually, cyclosporin A, a drug that binds to cyclophilin D, or diazoxide, a KATP opener, was able to significantly improve muscle function during the first contraction. Muscle function with cyclosporin A or diazoxide

aging the tissue, and causing subsequent muscular dysfunction (4, 33, 50). In our reoxygenation model, ROS levels were significantly increased in control images, suggesting antioxidant defenses were overwhelmed during the sudden influx of oxygen (Fig. 1). Therefore, it is possible that both XO and the mitochondrial complexes are potential sources of excessive levels of ROS production during reoxygenation in our study. Optimal amounts of ROS are involved in the protection of various tissues by different mechanisms. For instance, ROS production occurs during IPC, which invokes a cardioprotective effect responsible for mitigating necrotic cell death in the heart (12, 63). Accordingly, we propose that ROS are involved in an alternative mechanism that induces a protective effect against fatigue and dysfunction in skeletal muscle. Since muscle fatigue and cell death are very different physiological endpoints, the signaling pathways that lead to them will be markedly different although some similarities may be present. Previous research has shown that muscular contractions increase intra- and extracellular oxidants and free radicals contributing to muscle fatigue (47, 48). Therefore, small amounts of ROS generated from

Fig. 5. Data illustrating the average percentage of maximal force at the first and end contractions during the 15-min reoxygenation period compared with initial force measurements (control, n = 10; PO2 cycling, n = 7; 100 μM glibenclamide (KATP channel opening inhibitor) + PO2 cycling, n = 6; 50 μM carboxyatractyloside (ATP/ADP translocase inhibitor) + PO2 cycling, n = 5; 50 μM carboxyatractyloside (ATP/ADP translocase inhibitor) + PO2 cycling, n = 5; 100 μM glibenclamide (KATP channel opening inhibitor) + PO2 cycling, n = 5; 100 μM cyclosporin A (a drug that binds to cyclophilin D), n = 5; 100 μM diazoxide (KATP channel opener) + 50 μM carboxyatractyloside (ATP/ADP translocase inhibitor) + PO2 cycling, n = 5; 100 μM cyclosporin A (a drug that binds to cyclophilin D), n = 8]. Baseline force measurements were calculated before exposure to hypoxic and reoxygenation conditions. *P < 0.05, significantly different from control.
during the end contraction also trended towards enhanced performance under reoxygenation conditions. Yet, it was the combinatorial effects of diazoxide and cyclosporin A that produced a similar outcome to our PO2 cycling protocol: significant reduction in muscle fatigue during reperfusion for both first and end contractions (Figs. 3 and 5). The mPTP is sensitive to changes in the ATP/ADP translocase (23), also known as the adenine nucleotide translocator (28), which leads to mPTP activation via carboxyatractyloside. In addition, cyclophilin D is a positive modulator of mPTP opening and is inhibited when bound by cyclosporin A (13, 29). Thus our data indicate that both KATP and mPTP channels are integral components of the PO2 cycling protection pathway. Also, due to the relative ineffectiveness of cyclosporin A and diazoxide individually, it is possible that the two mitochondrial channels act in a synergistic manner. Although this connection has been previously described in the IPC mechanism, the current investigation has discovered a correlation between KATP and mPTP and the PO2 cycling pathway in skeletal muscle that has not been previously clarified. In addition, the results of our study suggest that individually diazoxide and cyclosporin A could not achieve the same effect on muscle fatigue resistance as the combinatorial effect of both agents. This is likely because, to achieve optimal protection, simultaneously opening KATP and closing mPTP channels by these agents are required to simulate PO2 cycling treatments. As shown in Fig. 6, our proposed PO2 cycling pathway includes these mitochondrial channels.

**Perspectives and Significance**

In this study, we examined the effectiveness of PO2 cycling in diaphragmatic skeletal muscle during reoxygenation and utilized in vitro muscle function data with mitochondrial and nonmitochondrial mediators to determine a potential protective mechanism. Although multiple studies have analyzed similar protocols such as IPC, which induces a cardioprotective effect to combat I/R injury, there is little research investigating nonpharmacological treatments in skeletal muscle tissue during I/R. Our data demonstrate a reduction in ROS levels and an increase in fatigue resistance in skeletal muscle fibers after PO2 cycling treatment in reoxygenation conditions. We have proposed a possible signaling cascade that may precipitate the PO2 cycling protective effect in skeletal muscle. This cycling may potentially be utilized to prevent this reoxygenation-related force loss in skeletal muscle. Clinical settings in which this preconditioning could potentially be of therapeutic value may include exhaustive exercise, mechanical ventilation, and frostbite. Our findings elaborate on important insights necessary to understand the molecular redox mechanism of PO2 cycling in diaphragmatic skeletal muscle during harmful reoxygenation conditions. Although we have determined the components of the PO2 cycling mechanism, the interactions among ROS, PI3K, Akt, ERK, KATP, and mPTP were not a part of our present study and have yet to be fully determined. Nonetheless, previous research has suggested that PI3K influences Akt and thus may also affect the expression of ERK (1, 3). Correspondingly, it has also been demonstrated that ERK plays a role in pathways that regulate the mitochondrial KATP and mPTP channels (37). Therefore, future research should focus on the precise interactions between these factors in skeletal muscle, in relation to each other and to ROS, to fully understand the PO2 cycling protection pathway. In summary, the results of our current study may have important implications in the development of nondrug therapies capable of alleviating hypoxia-reoxygenation related ailments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.Z. conception and design of research; L.Z. and B.K.P. performed experiments; L.Z., B.K.P., T.M.B., and P.D.W. analyzed data; L.Z., T.M.B., and P.D.W. interpreted results of experiments; L.Z., B.K.P., and T.M.B. performed figures; L.Z., B.K.P., T.M.B., and P.D.W. edited and revised manuscript; L.Z., B.K.P., T.M.B., and P.D.W. approved final version of manuscript.

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