Biochemical and Biophysical Research Communications 385 (2009) 28–32

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Role of mitochondrial ATP-sensitive potassium channels on fatigue in mouse muscle fibers

Maria C. García *, Ascención Hernández, Jorge A. Sánchez

Department of Pharmacology, Centro de Investigación y de Estudios Avanzados del I.P.N., 07360 Mexico City, DF, Mexico

ARTICLE INFO

Article history:
Received 19 April 2009
Available online 8 May 2009

Keywords:
Skeletal muscle
ROS
Tetanus
Force
Diazoxide
K_{ATP} channels
Fatigue
PKC

ABSTRACT

The role of mitochondrial K_{ATP} (mitoK_{ATP}) channels on muscle fatigue was assessed in adult mouse skeletal muscle bundles. Muscle fatigue was produced by eliciting short repetitive tetani. Isometric tension and the rate of production of reactive oxygen species (ROS) were measured at room temperature (20–22 °C) using a force transducer and the fluorescent indicator CM-H_2DCFDA. We found that opening mitoK_{ATP} channels with diazoxide (100 μM) significantly reduced muscle fatigue. Fatigue tension was 34% higher in diazoxide-treated fibers relative to controls. This effect was blocked by the mitoK_{ATP} channel blocker 5-hydroxydecanoate (5-HD), by the protein kinase C (PKC) inhibitor chelerythrine, and by the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) but was not accompanied by a change in the rate of ROS production during fatigue. A physiological role of mitoK_{ATP} channels on muscle fatigue is proposed.

Muscle fatigue is a physiological phenomenon in which a reversible decrease in tension is produced when skeletal muscles are used intensively. The decrease in force development is associated with multiple alterations at the cellular level, including changes in ion concentrations and cell metabolism, leading to a decrease in ATP during intense fatigue [1].

In excitable cells, ATP-regulated K^+ channels (K_{ATP} channels) play key roles linking excitability with metabolism. Although K_{ATP} channels were first identified in the cell membrane of cardiac muscle [2], K^+ flux experiments and the use of K^+ channel openers and blockers suggested the existence of a K_{ATP} channel in the inner membrane of mitochondria [3,4]. Opening of mitoK_{ATP} channels causes an increase in matrix volume leading to activation of the respiratory chain and an increase in ATP production [4,5]. It has been proposed that mitoK_{ATP} channels play a central role in ischemic preconditioning, an adaptive endogenous mechanism in heart and other tissues that protects them from severe damage after prolonged and intense ischemia. ROS generation is thought to be a trigger of signaling pathways mediating preconditioning [4,6]. However, the role of mitoK_{ATP} channels on other physiological phenomena like fatigue has not been explored.

The aim of the present study was to assess whether opening mitoK_{ATP} channels alters muscle fatigue and to determine whether ROS production is involved in this process. We found that the extent of fatigue is significantly decreased by mitoK_{ATP} channel openers, but no changes in the rate of ROS production during fatigue were observed.

Materials and methods

Animals and muscle preparation. The Animal Care Committee of Cinvestav approved all experimental procedures for this investigation. Adult male BALB/c mice were used. Mice were euthanized by cervical dislocation, and the *extensorum digitorum longus* (EDL) muscles were removed. Bundles of approximately 10–15 fibers were dissected from the lateral portion of the EDL, where fast muscle fibers are highly predominant [7].

Muscle chamber and solutions. Isometric force was measured as previously described [8]. A continuous flow of extracellular solution at a rate of ~0.7 ml/min was used. The physiological saline solution contained (in mM): 140 NaCl, 6 KCl, 2 $CaCl_2$, 3 $MgCl_2$, 5 Hepes, 11 glucose, and 0.025 *d*-tubocurarine chloride, at pH = 7.4. Diazoxide (a mitoK_{ATP} channel opener, 100 μM), 5-HD (a mitoK_{ATP} channel blocker, 125 μM), chelerythrine (a protein kinase C inhibitor, 1–1.5 μM), and L-NAME (a NO synthase inhibitor, 100 μM) were used. At these concentrations, these drugs are expected to have maximal effects on their targets [3,9–11]. Diazoxide and chelerythrine solutions were prepared by dissolving the drugs in DMSO and then adding the proper volume to the physiological solution. The final DMSO concentration was 0.1% (v/v). To test the actions of these compounds on fatigue, muscle bundles were preincubated

* Corresponding author. Fax: +52 55 5577 7090.
E-mail addresses: cgarcia@cinvestav.mx, garciag_mc@hotmail.com (M.C. García).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.05.019

for ~20–30 min (or 60–90 min with L-NAME) with the corresponding drug prior to applying the fatigue protocol. In experiments designed to test the effects of inhibitors in combination with diazoxide, muscle bundles were first preincubated with the inhibitor as described, and then diazoxide was added to the solution and incubated for a further 20–30 min. Experiments were conducted at 22–24 °C. This temperature range was used to preserve the stability of force over time as discussed elsewhere [12].

Stimulation protocol. Muscle bundles were stimulated with platinum electrodes using pulses of 1.5 ms at an intensity of 2–3× threshold (Digitimer model DS2A, England). The following stimulation protocol was applied: First, a single stimulus was delivered to produce a twitch. This was followed by a 300 ms tetanus at the frequency of 45 Hz, elicited 200 ms after the twitch. This stimulation pattern was repeated every 4 min to record tension under conditions that do not lead to fatigue. Next, fatigue was produced by applying the same stimulation pattern at 3-s intervals for 6 min. Finally, recovery from fatigue was monitored by applying twitch–tetanus cycles at 0.5, 1–5 min after fatigue and then every 4 min over ~30–35 min.

Force measurement. Forces were measured with a Cambridge transducer (model 400A) and digitized with an analog-to-digital converter (Digidata 1322A, Molecular Devices, USA). Maximum twitch and tetanic forces were measured after subtraction of the baseline. A fatigue index was calculated as the ratio between peak tetanic force produced at the end of the 6 min stimulation period and the corresponding value at the beginning of the stimulation protocol.

Measurement of ROS production. The generation of ROS was measured using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Molecular Probes, Oregon, USA). CM-H_2DCFDA is cell permeant, and intracellular esterases cleave the diacetate group to trap the non-fluorescent product CM-H_2DCFFH [13]. Upon exposure to H_2O_2 or ·OH the probe is oxidized to CM-H_2DCF [13] and becomes fluorescent; its intensity depends on the amount of ROS generated. The probe has excellent retention in live cells and has been used to measure intracellular ROS in cardiac myocytes and other cells [14]. Fluorescence (in arbitrary units) was measured from user-defined segments of single muscle fibers over 200 ms at excitation and emission wavelengths of 470 nm and 515 nm, respectively. The intensity of the emitted light was detected with a low-noise photodiode connected in a photovoltaic configuration. To avoid mechanical artifacts, measurements were not made during muscle contraction. The rate of increase in fluorescence over time, representing the rate of ROS production, was determined by calculating the slope of consecutive fluorescence signals. Loading of muscle cells with 2.5 μM CM-H_2DCFDA was achieved by incubation of the probe at room temperature for 30 min in the dark. After loading, cells were incubated in a dye-free solution for over 30 min to allow the conversion of the dye into its ROS-sensitive form. At the end of experiments, loaded fibers were exposed to constant excitation for 3–6 min to induce the photo-oxidation of all available CM-H_2DCFDA in the fibers; fluorescence values increased with light exposure until they reached a plateau that represented the total amount of CM-H_2DCFDA available for oxidation within the exposed area of the fiber.

Statistical analysis. Student's *t*-test or ANOVA was used as required. Values of $p < 0.05$ were considered statistically significant.

Results and discussion

MitoK_{ATP} channel openers reduced fatigue

Selective blockers and openers of mitoK_{ATP} channels have been used extensively to investigate mitoK_{ATP} channel function [3,9]. Diazoxide is a very selective opener of mitochondrial K_{ATP} channels; at a concentration of 100 μM it fully opens mitoK_{ATP} channels with no effect on sarcolemmal K_{ATP} channels [3] or the current–voltage relationship [15]. In skeletal muscle, the effect of diazoxide on the respiratory rate of isolated mitochondria is maximal at this concentration [16]. In the present study, we used diazoxide (100 μM) and found that it significantly reduced fatigue. Fig. 1A shows a continuous recording of the tension generated during twitches and tetani by a bundle of EDL skeletal muscle fibers. Intermittent stimulation led to muscle fiber fatigue, manifested by an initially rapid and then slower progressive fall in tetanic tension to a fraction of the initial force. The inset in Fig. 1A illustrates the first and last force recordings during the fatigue protocol in an expanded time scale. The dotted line represents the average value of tetanic tension in fatigued fibers from similar experiments. When the same protocol was applied in the presence of diazoxide, muscle fibers also experienced fatigue, but the decline in tension was distinctly smaller at every time point, as shown in a separate

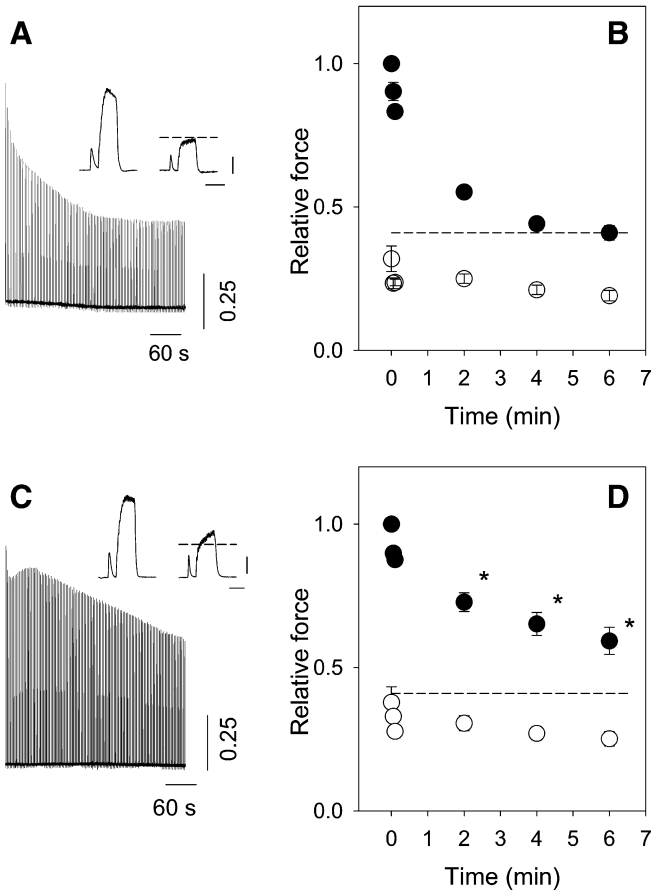

Fig. 1. Diazoxide reduced fatigue in mouse muscle fibers. (A) Shows a continuous recording of twitch and tetanic tension from an EDL muscle bundle under control conditions. The vertical calibration bar is tension relative to the first tetanus. The inset illustrates the first and last force recordings from the same experiment. Calibration bars are 300 ms and 0.2. (B) Shows the development of fatigue over time. Each data point represents average values ± SE ($n = 25$) under control conditions of twitch (○) and tetanic (●) tension as a function of time (min). Force is expressed relative to tetanic tension at t = 0 min. The horizontal dotted line represents the average value of relative tetanic tension at $t = 6$ min. (C) Shows fatigue results from a separate experiment in the presence of diazoxide. The fatigue protocol was as in (A). Calibration bars are as in (A). (D) Shows the development of fatigue over time in the presence of diazoxide. Each data point represents average values ± SE ($n = 15$) from diazoxide-treated fibers of twitch (○) and tetanic (●) tension as a function of time. Force is expressed relative to tetanic tension at $t = 0$ min. The dotted lines in (C) and (D) were taken from (A) and (B) to facilitate visual comparison with control data. * $p < 0.05$ vs. control.

experiment in Fig. 1C, suggesting that opening mitoK$_{ATP}$ channels reduced fatigue. The inset in Fig. 1C illustrates the first and last tension recordings during the fatigue protocol from the same experiment. Tetanic force declined less than in control experiments, as indicated by the dotted line that represents the average value of tetanic force in fatigued fibers under control conditions, taken from (A). We verified that diazoxide by itself did not affect twitch or tetanic tension. Thus, the mean force (in milligrams) during the first tetanus averaged 46.9 ± 11.2 (n = 25) in control experiments and 47.0 ± 8.6 (n = 15) in diazoxide-treated muscle bundles. Fig. 1B and D summarize the effect of diazoxide on fatigue. Illustrated are average values of relative twitch (○) and tetanic tension (●) as a function of time, computed from the onset of the fatigue protocol. Values were normalized to the peak tension developed during the first tetanus. Under control conditions (Fig. 1B), tetanic tension (●) decreased to almost half of the initial force in 2 min, approaching an asymptotic value of 0.4 at 6 min (dotted line). Twitch tension (○) also decreased with fatigue. At the end of the fatigue protocol, twitch tension fell 40% from its initial value. In diazoxide-treated fibers, the decline in tetanic tension (●) over time was smaller (Fig. 1D). To facilitate visual comparison between the data from control and from diazoxide-treated fibers, the dotted line from Fig. 1B was replotted in (D). At the end of the fatigue protocol, tetanic force decreased to 59% of its initial value, indicating less fatigue. The fall in twitch tension (○) was less pronounced, falling to 66% of its initial value.

The experiments in Fig. 1 are clearly consistent with the view that diazoxide attenuates fatigue by opening mitoK$_{ATP}$ channels. Additional evidence for this hypothesis was provided by the use of a specific blocker for this channel. If diazoxide antagonizes fatigue by opening mitoK$_{ATP}$ channels, it would be expected that addition of 5-HD, a selective blocker [4], would prevent the effect of diazoxide on fatigue. We found that this is indeed the case. This is shown in Fig. 2A, which illustrates the first and last recordings taken during the fatigue protocol from the same muscle bundle. Data were normalized to the maximum tension generated during the first tetanus. The decline in tetanic tension in the presence of diazoxide plus 5-HD was similar to that observed in control experiments. The dotted line in this and subsequent panels represents the average value of tetanic tension developed by fatigued fibers under control conditions taken from Fig. 1A. We next tested chelerythrine, an inhibitor of protein kinase C. Previous work has shown that this kinase is implicated in mitoK$_{ATP}$ channel function [17]. The δ isoform of PKC is located in the inner mitochondrial membrane, and PMA, a phorbol ester that activates protein kinase C, increases the open probability of single cardiac mitoK$_{ATP}$ channels [18]. Therefore, it would be expected that inhibition of PKC would close mitoK$_{ATP}$ channels, preventing the action of diazoxide. We indeed found that the inhibitory action of diazoxide on fatigue was completely eliminated by chelerythrine (Fig. 2B). Finally, to further assess whether mitoK$_{ATP}$ channels are involved in the diazoxide effect, we tested L-NAME, an analog of arginine that inhibits NO synthase. NO, an important messenger molecule in cells, is formed in skeletal muscle during contractions [19], and it regulates mitoK$_{ATP}$ channel function in other systems. For example, Ockaili et al. [20] showed that the cardioprotective effects of diazoxide *in vivo* are blocked by L-NAME. Furthermore, there is evidence indicating that NO directly activates mitoK$_{ATP}$ channels [21,22]. We found that fatigue was similar to controls in muscle fibers incubated with diazoxide plus L-NAME (Fig. 2C), consistent with a role of mitoK$_{ATP}$ channels in fatigue.

Fig. 2D summarizes the effects of diazoxide and mitoK$_{ATP}$ channel inhibitors on fatigue. Diazoxide significantly reduced the extent of fatigue during muscle activity, an effect that was completely blocked by the mitoK$_{ATP}$ channel blocker 5-HD, chelerythrine, and L-NAME. These inhibitors by themselves had no effect on

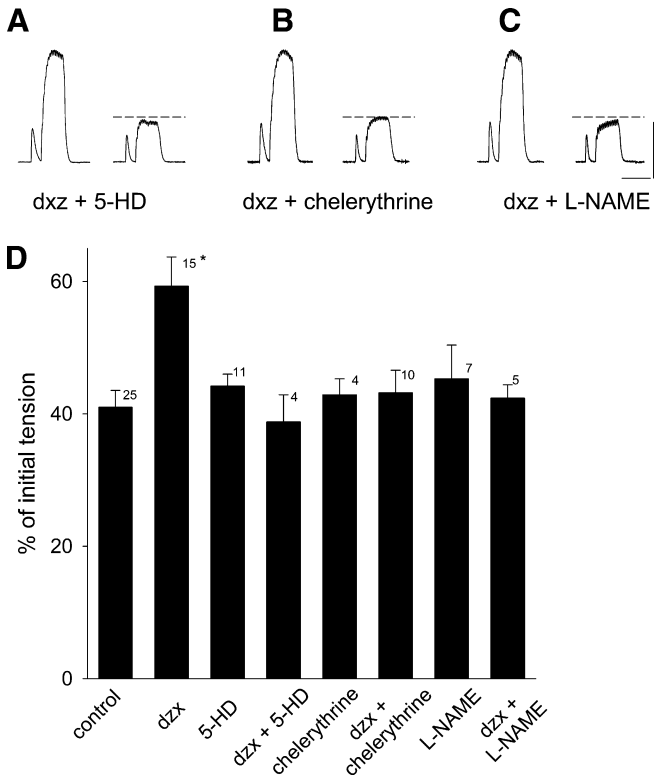

Fig. 2. The effect of diazoxide on fatigue in the presence of inhibitors. (A–C) Illustrate recordings of the first and last cycles of twitch and tetanic tension from EDL muscle bundles during the fatigue protocol. Each panel shows a separate experiment in the presence of diazoxide (dxz) plus the indicated inhibitor. The horizontal dotted line represents the average value of relative tetanic tension at the end of the fatigue protocol from control experiments. Tension was normalized relative to the first tetanus. Calibration bars are 350 ms and 0.5. In (D), each bar represents the average (±SE) relative tetanic tension recorded at the end of the fatigue protocol. Fatigue was partially inhibited in the presence of diazoxide (dzx), an effect that was blocked by 5-HD, chelerythrine and L-NAME. The number of experiments is indicated above each bar. $^{*}p < 0.05$ vs. control.

fatigue. It should be pointed out that although diazoxide and 5-HD have mitoK$_{ATP}$ channel-independent targets [23,24], our results, taken together, suggest that diazoxide antagonizes muscle fatigue through signaling pathways involving PKC, probably leading to the activation of the NO synthase with subsequent synthesis of NO and opening of the mitoK$_{ATP}$ channel.

ROS production and mitoK$_{ATP}$ channels

Force and the rate of ROS production were measured in the same muscle bundles, and the effect of opening mitoK$_{ATP}$ channels with diazoxide on both parameters was assessed. Fig. 3A and B show the force generated by muscle bundles stained with CM-H$_2$DCFDA. (A) Illustrates a control experiment and (B) shows an experiment in the presence of diazoxide. In each panel, the *initial* and the *fatigue* traces correspond to the first and last recordings made during the fatigue protocol, respectively. The *recovery* trace was taken when fibers recovered from fatigue, ~4–5 min after the end of the fatigue protocol. Consistent with the experiments illustrated in Figs. 1 and 2, diazoxide significantly reduced the fall of twitch and tetanic tension in fatigued fibers. To assess the possible involvement of ROS on the diazoxide response, the rate of ROS production was first measured under non-fatigued conditions. Next, the fatigue protocol was applied, and the rate of ROS production was measured during fatigue and within the first 3–5 min of recovery. Fig. 3C summarizes results from several experiments. In control experiments, the rate of ROS production during fatigue (f)

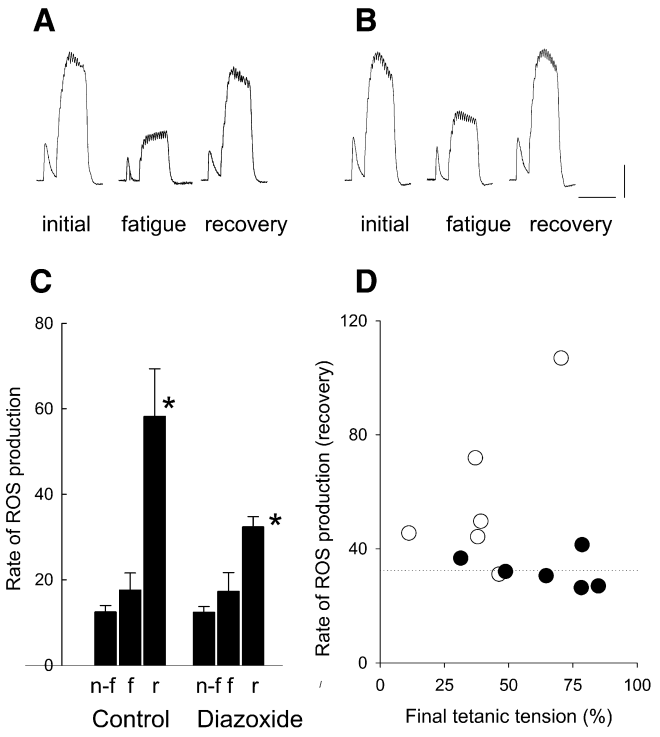

Fig. 3. Rate of ROS production and fatigue. (A) Shows representative recordings of control twitch and tetanic tension at the beginning and end of the fatigue stimulation protocol and after recovery. (B) Demonstrates the corresponding recordings in the presence of diazoxide. Tension was normalized relative to the first tetanus. Calibration bars are 400 ms and 0.25. (C) Shows the average rate of ROS production (in arbitrary units) ± SE from experiments similar to those in (A) and (B) (*n-f*, non-fatigue; *f*, fatigue; *r*, recovery). $*p < 0.05$ vs. control, $n = 6–7$. (D) Shows the rate of ROS production in arbitrary units as a function of the normalized tetanic tension at the end of the fatigue protocol. Each data point represents a separate experiment under control conditions (○) and in the presence of diazoxide (●). The dotted line is the average value of the rate of ROS production from diazoxide-treated cells.

did not increase significantly beyond the level measured under non-fatigued conditions (*n-f*). This observation is consistent with the findings of Bruton et al. [12] who described that the myoplasmic ROS concentration in mouse muscle fibers remains unchanged during fatiguing stimulation. Furthermore, the number of tetani required to decrease force does not change in the presence of the ROS scavenger NAC [12].

On the other hand, we found that the rate of ROS production increased quite significantly during recovery (*r*), reaching a value more than three times that of non-fatigued fibers. This is in concordance with previous reports showing that ROS production increases after mechanical activity [19,25,26]. The mechanism is unclear, but it has been shown that intracellular pH in skeletal muscle decreases and phosphocreatine is resynthesized after exercise [1]. A decrease in pH and other exercise-associated changes stimulate intracellular oxidant activity in muscle fibers [27].

We found that the rate of ROS production in muscle fibers incubated with diazoxide under non-fatigue and fatigue conditions was similar to controls, but that the increase in the rate of ROS production during recovery from fatigue was greatly reduced (Fig. 3C). This observation is illustrated in more detail in Fig. 3D. The rate of ROS production during recovery from fatigue was plotted as a function of the normalized tetanic tension at the end of the fatigue protocol. In control experiments (○), the rate of ROS production during recovery clearly depended on the extent of fatigue achieved. However, in the presence of diazoxide, the rate of ROS production during recovery from fatigue (●) was distinctly lower and did not vary with the tension reached at the end of fatigue. The dotted line represents the average value of the rate of ROS production in diazoxide-treated cells. The effect of diazoxide on the rate of ROS production during recovery from fatigue is consistent with the observation that opening mitoK_{ATP} channels decreases ROS generation in isolated heart mitochondria [28].

Acknowledgments

We thank Drs. Sergio Sánchez-Armass and Rodrigo Sánchez for critically reviewing this manuscript and Eva Sandoval and Oscar Ramírez for technical assistance. This work was supported in part by CONACYT Grants 82667 and 60880.

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