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Original Contribution

The anticancer agent doxorubicin disrupts mitochondrial energy metabolism and redox balance in skeletal muscle

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ABSTRACT

The combined loss of muscle strength and constant fatigue are disabling symptoms for cancer patients undergoing chemotherapy. Doxorubicin, a standard chemotherapy drug used in the clinic, causes skeletal muscle dysfunction and premature fatigue along with an increase in reactive oxygen species (ROS). As mitochondria represent a primary source of oxidant generation in muscle, we hypothesized that doxorubicin could negatively affect mitochondria by inhibiting respiratory capacity, leading to an increase in H_2O_2-emitting potential. Here we demonstrate a biphasic response of skeletal muscle mitochondria to a single doxorubicin injection (20 mg/kg). Initially at 2 h doxorubicin inhibits both complex I- and II-supported respiration and increases H_2O_2 emission, both of which are partially restored after 24 h. The relationship between oxygen consumption and membrane potential ($\Delta\Psi$) is shifted to the right at 24 h, indicating elevated reducing pressure within the electron transport system (ETS). Respiratory capacity is further decreased at a later time point (72 h) along with H_2O_2-emitting potential and an increased sensitivity to mitochondrial permeability transition pore (mPTP) opening. These novel findings suggest a role for skeletal muscle mitochondria as a potential underlying cause of doxorubicin-induced muscle dysfunction.

Introduction

Doxorubicin is a potent anthracycline antibiotic used to treat numerous human malignancies [1]. A severe side effect of doxorubicin is cardiotoxicity characterized by a dose-dependent decline in cardiac function with prolonged exposure [2]. Clinicians manage this side effect by limiting the dosage patients receive; however, even on a limited dose patients can experience disabling muscle weakness and fatigue [3,4]. In the clinic, fatigue is generally documented as perceived fatigue, or a sense of tiredness, which is difficult to distinguish from physiological fatigue [5]. Physiological fatigue involves muscle-specific peripheral fatigue, which includes two components: muscle fatigue and muscle weakness. Our previous work suggests that the decline in muscle function observed in patients could be due to an effect specifically on skeletal muscle. Healthy rodents exposed to a clinical dose of doxorubicin exhibit a decrease in both hindlimb and respiratory muscle strength, along with an accelerated rate of fatigue [6–8]. The loss of strength in combination with constant fatigue can burden patients, not only during therapy, but up to 10 years following the cessation of therapy [9].

Potential mediators of doxorubicin-induced muscle weakness and fatigue are reactive oxygen species (ROS). In cardiac muscle, doxorubicin is known to increase ROS by localizing to the mitochondria [10] where it is reduced by complex I to form a semiquinone radical [11]. In addition, indirect ROS production can occur with doxorubicin through inhibition of the mitochondrial electron transport system (ETS). Previous reports demonstrate that doxorubicin inhibits the ETS in isolated heart mitochondria, specifically complexes I and II [12,13]. An overproduction of oxidants due to a block in the ETS can lead to redox modifications of cell macromolecules (e.g., proteins, lipids, DNA) with detrimental downstream effects on whole organ function [14,15].

In skeletal muscle, cytosolic oxidant activity and markers of protein oxidation are elevated following doxorubicin exposure [7,16]. Mitochondria represent a primary source of oxidant generation in skeletal muscle [17,18]. Doxorubicin-induced oxidants are blunted in C2C12 myotubes following incubation with Bendavia (Stealth Peptides, Newton, MA; formerly known as SS31) [19], a cell-permeable peptide that localizes to the mitochondria and lessens ROS production. These findings suggest that doxorubicin

Abbreviations: ETS, electron transport system; mPTP, mitochondrial permeability transition pore; PmFBs, permeabilized fiber bundles; ROS, reactive oxygen species; TPP, tetraphenylphosphonium

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could be affecting skeletal muscle mitochondrial function, leading to ROS production.

The objective of this study was to determine the exact nature and extent to which mitochondrial function is impacted by doxorubicin treatment, specifically in skeletal muscle. It was hypothesized that doxorubicin would inhibit skeletal muscle mitochondrial respiration, leading to an increase in ROS-emitting potential. To test this hypothesis mitochondrial function was evaluated in permeabilized fiber bundles (PmFBs) 2, 24, and 72 h following a single doxorubicin injection (20 mg/kg). The results indicated a biphasic response. Doxorubicin initially (2 h) induced an increase in H_2O_2 emission and membrane potential with a decline in respiratory function that was reversed after 24 h. After 72 h, respiratory capacity was again decreased along with H_2O_2-emitting potential and membrane potential, indicative of a decline in overall mitochondrial function.

Methods

Overview of experimental design

Male Sprague-Dawley rats (Charles River Laboratories) 8–10 weeks old (~250 g) received an intraperitoneal injection of doxorubicin (20 mg/kg in phosphate-buffered saline), a clinically applicable dose that is equivalent to what patients with hematological malignancies receive [20], based on the conversion factor established by Freireich et al. [21]. Control animals received the same volume of vehicle (PBS). Following injection, rats were housed in metabolic chambers for 72 h for indirect open circuit calorimetry measurements. Rats were monitored and weighed daily. PmFBs were prepared from the red gastrocnemius muscle at three different time points postinjection: 2 h (n= 8 for CTRL; n=8 for DOX), 24 h (n=10 for CTRL; n=6 for DOX), and 72 h (n=15 for CTRL; n=13 for DOX).

Rodent care and reagents

All rodents were housed in the Department of Comparative Medicine at East Carolina University in a temperature- and light-controlled room and given free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee. Skeletal muscle was obtained from anesthetized rats (100 mg/kg intraperitoneal ketamine-xylazine). Following surgery, rats were euthanized by cervical dislocation under anesthesia. Doxorubicin was purchased from Bedford Laboratories (Bedford, OH). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The Total OxPhos Complex Kit used to measure mitochondrial content was purchased from Invitrogen (Frederick, MD). Fluorescence-conjugated secondary antibodies were purchased from LI-COR (Lincoln, NE).

Determination of body composition

Pre- and postinjection (72 h) measurements of fat and lean body mass were determined using the EchoMRI-500 (Houston, TX) in accordance with the manufacturer's instructions.

Indirect in vivo metabolic parameters

The TSE LabMaster System (TSE Systems, Chesterfield, MO) was used to determine rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2), respiratory exchange ratio, food and water intake, and energy expenditure. Energy expenditure was calculated by the system using the equation $((CVO_2 * VO_2)+(CVCO_2 * VCO_2)/1000)$. Constants of the equation include: CVO_2=3.941 [ml/h] and $CVCO_2$= 1.106 [ml/h]. All rates are expressed per gram of body weight determined every 24 h. Infrared sensors were used to record ambulatory activity in three-dimensional axes (*X, Y, Z*). Counts across all three axes were summed to give total ambulatory activity.

Permeabilized fiber bundle preparation

Procedures were performed as described previously [22–24]. In brief, fiber bundles from the red portion of the gastrocnemius muscle were separated with fine forceps in ice-cold Buffer X (in mM: 50 K-MES, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 20 taurine, 5.7 ATP, 14.3 PCr, 6.56 $MgCl_2$-6 H_2O; pH 7.1). After separation, fiber bundles were permeabilized in Buffer X with 40 μg/ml saponin for 30 min and then washed in ice-cold Buffer Z (in mM: 105 K-MES, 30 KCl, 1 EGTA, 10 K_2HPO_4, 5 $MgCl_2$-6 H_2O, 0.5 mg/ml BSA; pH 7.1) until analysis. A subset of fibers was exposed (15 min) to dithiothreitol (DTT, 1 mM in Buffer Z) following permeabilization before mitochondrial function analysis.

Mitochondrial respiration

High-resolution O_2 consumption measurements were conducted using the OROBOROS Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) at 37 °C with an initial chamber concentration of 300–350 μM oxygen. This concentration does not alter respiratory kinetics [22] and maximizes the duration of experiments before oxygen becomes rate limiting. All experiments were run in Buffer Z containing 20 mM creatine monohydrate and 25 μM blebbistatin (myosin II ATPase inhibitor). Individual protocols included: (1) 2 mM malate+4 mM ADP followed by sequential additions of glutamate up to 16 mM; (2) 15 mM glutamate+2 mM malate followed by sequential additions of ADP up to 4 mM; (3) 2 mM malate+4 mM ADP followed by sequential additions of pyruvate up to 4 mM; (4) 5 mM pyruvate+2 mM malate followed by sequential additions of ADP up to 4 mM; (5) 10 μM rotenone+4 mM ADP followed by sequential additions of succinate up to 14 mM; (6) 2 mM malate+4 mM ADP followed by sequential additions of palmitoylcarnitine up to 75 μM. The K_m was determined through the Michaelis-Menten enzyme kinetics using Prism Graphpad (La Jolla, CA). At the end of each protocol cytochrome *c* was added to test for mitochondrial membrane integrity. Any PmFB that generated a >10% increase in respiration following the addition of cytochrome *c* was not included in the data analysis. At the conclusion of each experiment, PmFBs were washed in dH_2O and dried via freeze-drying (Labconco, Kansas City, MO). Polarographic oxygen measurements are expressed as picomoles per second per milligram dry weight.

Mitochondrial H_2O_2 emission

H_2O_2 emission was measured at 37 °C in Buffer Z containing 5000 U/ml CuZn-SOD, 25 μM blebbistatin, 50 μM Amplex UltraRed (AUR), and 6 U/ml horseradish peroxidase. Resorufin fluorescence (peroxidation product of AUR) was monitored by the Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) at excitation/emission 568/581 nm. Individual protocols included: (1) 9 mM succinate; (2) 10 mM pyruvate+2 mM malate+10 mM glutamate; (3) 25 μM palmitoyl-L-carnitine+10 mM glycerol-3-phosphate+10 mM antimycin A. For each protocol a background fluorescence rate was established in the presence of the PmFB, followed by the addition of subsequent substrates. After correcting for the rate of change in background fluorescence, the concentration of H_2O_2 (pmol) was calculated from previously established resorufin fluorescence intensity standard curves with known concentrations of H_2O_2 for each individual protocol. Following each experiment, PmFBs were dried and weighed (as described above)