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Reductive stress impairs myoblasts mitochondrial function and triggers mitochondrial hormesis

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ABSTRACT

Even though oxidative stress damage from excessive production of ROS is a well known phenomenon, the impact of reductive stress remains poorly understood. This study tested the hypothesis that cellular reductive stress could lead to mitochondrial malfunction, triggering a mitochondrial hormesis (mitohormesis) phenomenon able to protect mitochondria from the deleterious effects of statins. We performed several in vitro experiments on L_6 myoblasts and studied the effects of N-acetylcysteine (NAC) at different exposure times. Direct NAC exposure (1 mM) led to reductive stress, impairing mitochondrial function by decreasing maximal mitochondrial respiration and increasing H_2O_2 production. After 24 h of incubation, the reactive oxygen species (ROS) production was increased. The resulting mitochondrial oxidation activated mitochondrial biogenesis pathways at the mRNA level. After one week of exposure, mitochondria were well-adapted as shown by the decrease of cellular ROS, the increase of mitochondrial content, as well as of the antioxidant capacities. Atorvastatin (ATO) exposure (100 μM) for 24 h increased ROS levels, reduced the percentage of live cells, and increased the total percentage of apoptotic cells. NAC exposure during 3 days failed to protect cells from the deleterious effects of statins. On the other hand, NAC pretreatment during one week triggered mitochondrial hormesis and reduced the deleterious effect of statins. These results contribute to a better understanding of the redox-dependant pathways linked to mitochondria, showing that reductive stress could trigger mitochondrial hormesis phenomenon.

1. Introduction

The mitochondrion is the most important organelle in determining continued cell survival and cell death. In most cell types, mitochondria are the major source of reactive oxygen species (ROS), which affect apoptosis both directly and indirectly via the activation of transcription factors [1]. For a long time, ROS were considered exclusively unwanted by-products of oxidative phosphorylation, as high concentrations of ROS cause lipid peroxidation and damage to cell membranes, proteins, carbohydrates, and DNA [2]. In the last decade, it has been shown that low, non-cytotoxic concentrations of ROS can serve as signals, triggering the activation of specific pathways [3–10]. The preservation of this non-cytotoxic level of ROS is ensured by antioxidant systems, which play a major role in cellular redox homeostasis. A deficiency or defect in these systems leads to an increase in tissue damage. In such cases, ROS can act either as second messengers or as a source of cellular damage, depending on the level of ROS production [4,6,7,11]. The concept of mitochondrial hormesis, or "mitohormesis", proposes that low doses of mitochondrial ROS can activate mitochondrial biogenesis and antioxidant capacities in order to counteract oxidative stress and to re-establish homeostasis [4,5,7,8,12].

The development of chronic oxidative stress has been implicated in the metabolic myopathy that is a secondary symptom of numerous pathologies such as diabetes mellitus, heart failure, or chronic obstructive pulmonary disease (COPD) [13,14]. This myopathy is also the most common adverse event encountered in patients treated with statins (HMG-CoA inhibitors, used to lower the plasmatic cholesterol levels), the most frequently prescribed treatment in developed countries. Recently, we found that statins protect mitochondria in the highly oxidative cardiac muscle by triggering a mitohormesis mechanism but impair mitochondrial function in glycolytic skeletal muscle [4].

Therefore, strategies involving moderate mitochondrial stress, inducing a mitohormesis mechanism may be used for the development of new ROS-targeting drugs. This would result in reducing the symptoms of metabolic myopathies by strengthening muscular mitochondrial function [11,15].

Even though oxidative stress damage from excessive production of ROS is a well known phenomenon, the impact of reductive stress

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remains poorly understood. Reductive stress can be defined as an excess of reducing equivalents (NAD(P)H and/or GSH) in the presence of intact oxido-reductive systems [2]. N-acetylcysteine (NAC) is the acetylated form (efficiently metabolized) of cysteine and is the most immediate precursor of glutathione (GSH). The GSH system is the main mechanism of detoxification of free radicals and ionized metabolites [16,17], and it is partly localized in the mitochondria. NAC is mainly used for its mucolytic action on the disulfide bonds of mucoproteins [18] and its modulating action on oxidative stress [19]. Interestingly, it has been shown that NAC treatment in H_9C_2 cells led to reductive stress [20]. This condition paradoxically increased the level of mitochondrial oxidation. However, the consequences as well as the potential beneficial effects following this reductive stress-inducing mitochondrial oxidation remained undiscovered [2].

We hypothesized that NAC treatment could change the redox environment of the cell, inducing mitochondrial oxidation (as described by [2,20]) and triggering a mitohormesis phenomenon, protecting L_6 myoblasts from the deleterious effects of statins.

Therefore, we investigated the effects of N-acetylcysteine on L_6 myoblasts. We found that NAC induced cellular reductive stress, which had for consequence an inhibitory effect on the mitochondrial respiratory chain, leading to an increase in mitochondrial ROS production and mitochondrial oxidation. This triggered mitochondrial biogenesis pathways and led to an increase in the number of mitochondria and in antioxidant capacities after 7 days of incubation. Finally, we showed that mitochondrial hormesis, triggered by NAC, protected cells against statin-induced apoptosis.

2. Materials and methods

2.1. Cell culture

L_6 rat myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM Low glucose, Milerium, VWR International) supplemented with 20% fetal calf serum (FCS) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco) at 37 °C under a humidified 5% CO_2 atmosphere. These cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

The acute effects of N-acetylcysteine (Sigma) were studied at several doses (50 μM, 500 μM, 1 mM, and 5 mM) for mitochondrial respiration, H_2O_2 production, and for the detection of superoxide production by electron spin resonance (ESR).

The chronic effects of N-acetylcysteine on L_6 myoblasts were studied by incubation with 1 mM of NAC in the culture medium for 24 h, three days, and one week. For the three days and one-week experiments, cells were also incubated with 100 μM atorvastatin for the last 24 h, in DMEM containing 10% FCS and 1% antibiotics.

2.2. Cell viability assays

Cell viability assays were performed after 24 h or one week of incubation with or without 1 mM NAC in DMEM containing 20% FCS, using a Muse™ Cell Analyzer (Merck Millipore), with the Muse Count and Viability Assay kit (Merck Millipore, Cat. Number: MCH100102), following the manufacturer's indications.

2.3. Study of mitochondrial respiration by oximetry

Mitochondrial respiration was studied in saponin-skinned cells to keep mitochondria in their architectural environment [4]. The analysis took place in a thermostated oxygraphic chamber at 37 °C with continuous stirring (Oxygraph-2 k, Oroboros instruments, Innsbruck, Austria). Cells were collected with trypsin and placed in R^+ medium (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 6.56 mM $MgCl_2$, 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 50 mM K-methane sulfonate, 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/ml of BSA; pH = 7) in the oxygraphic chamber with saponin (0.125 mg/ml per chamber). Maximal respiration rate (V_{max}) was measured in the presence of saturating amounts of succinate (25 mM) and ADP (2 mM) as substrates. V_{max} characterizes the electron flow through complexes I, II, III, and IV. NAC was directly injected into the oxygraphic chamber after V_{max}. After the experiments, cells were collected for total protein content determination. Results are expressed as a percentage of the V_{max} or of the control group.

2.4. H_2O_2 production in permeabilized cells

H_2O_2 production was measured with the Amplex Red reagent (Invitrogen), which reacted with H_2O_2 in a 1:1 stoichiometry catalyzed by HRP (Horse Radish Peroxidase; Fluka Biochemika) to yield the fluorescent compound resorufin and molar equivalent O_2 [21]. Resorufin has excitation/emission characteristics of 563/587 nm and is extremely stable once formed. Fluorescence was measured continuously [change in fluorescence (F)/s] with a Fluoromax-4 (Jobin Yvon) spectrofluorometer with temperature control (set at 37 °C) and under continuous magnetic stirring.

Approximately 300,000 cells were added to 600 μl of buffer Z (110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K_2HPO_4, 3 mM $MgCl_2$, and 0.5 mg/ml BSA) with HRP (0.5 U/ml) and Amplex Red (5 μM). Saponin was directly added to allow cell permeabilization (0.125 mg/ml saponin). H_2O_2 production was then measured with glutamate (5 mmol/l), malate (2 mmol/l), succinate (25 mM), and ADP (2 mM) to stimulate electron flow through complexes I, II, III, and IV. To study the acute effects of NAC, 1 mM of NAC was then added in the spectrofluorometer's quartz cell. H_2O_2 production rate was calculated from the slope of F/s, after subtracting background, from a standard curve established with the appropriate reaction conditions. At the conclusion of each experiment, cells were collected for total protein content determination. Values are expressed as percentage of the control group.

2.5. Mitochondrial free radical leak (FRL)

H_2O_2 production and O_2 consumption were measured in parallel in the same sample under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence which reduce O_2 to ROS in the respiratory chain (the percentage of free radical leak) instead of reaching cytochrome oxidase to reduce O_2 to water [21]. Because two electrons are needed to reduce 1 mol of O_2 to H_2O_2, whereas four electrons are transferred in the reduction of 1 mol of O_2 to water, the percent of FRL was calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption, and the result was multiplied by 100.

2.6. NAD^+/NADH determination

NAD^+/NADH ratio was determined after 1H incubation with NAC 1 mM, using the EnzyChrom™ NAD^+/NADH assay kit (BioAssay Systems, Cat. Number: E2ND-100), following the manufacturer's instructions.

2.7. Electron spin resonance measurement of superoxide production

Superoxide ($O_2^{\bullet -}$) production was determined after incubation with 1 mM NAC for either 24 h, three days, or one week. For the three days and one-week experiments, cells were also incubated with 100 μM atorvastatin for the last 24 h, in DMEM containing 10% FCS and 1% antibiotics. Cells were placed into a 24-well plate with Krebs–Hepes Buffer containing 25 μmol/l deferoxamine and 5 μmol/l DETC. Cells were then incubated at 37 °C with the spin probe CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine HCl, 200 μM) for 30 min under 2.7% oxygen, and 20 mm Hg partial pressure using a Gas-Controller (Noxygen Sciences Transfer, Elzach, Germany). The reaction was then stopped on ice. All ESR experiments measuring the