Oxygen consumption of yeast cell mitochondria in the presence of antioxidants to determine the role of reactive oxygen species and superoxidase dismutase

Abstract

Antioxidants play an important role in mitochondrial function in the presence of reactive oxygen species (ROS). For example deficiency of the antioxidant glutathione has been shown to cause oxidative stress in different types of dialysis patients (Ross). ROS are naturally produced as byproducts of various cellular processes such as respiration and develop in greater amounts under situations of environmental stress. Common ROs include radical dotO2- , radical dotO2-2, H2O2, radical dotOH, and OH-. The dot radical dot represents an unpaired electron in various formulas (Held).

This experiment was performed to determine the role of reactive oxygen species in the mitochondria of yeast cells. Yeast solutions were prepared with dextrose and broth to allow respiration to occur. Mitochondria were isolated from yeast cells that were mixed with quercetin and without quercetin. Through two different methods: mechanical isolation and enzymatic breaking down of the cell membranes, the process involved a series of centrifugation and homogenization (Qiagen). The mitochondria were immersed in a mitochondrial suspension solution with a pyruvate-malate solution and their oxygen consumption rate was measured with and without the presence of ADP. The pyruvate-malate solution served to stimulate the Citric acid cycle. A graph of the percentage of oxygen in the mitochondria sample was generated with two distinct slopes: one before (stage IV) and one after (stage III) ADP injection. The ratio between these two slopes and the ratio of phosphorous to oxygen in the sample determines the ability of the mitochondria to function.

The pathways involved in the reduction of oxygen molecules during the flow of the electron transport chain are being studied. The gene(s) that cause the formation of the antioxidants have yet to be identified. They could be from the nucleus or the mitochondria. The links between the ROS and the “protective” mechanisms and “damage” exposure in the mitochondria are being studied, and the ways to increase mitochondrial biogenesis are being studied. The role the ROS play in oxidative damage of aging could further be investigated (Sohal). For further research, mice could be tested and NMR could be used to determine the structure of the mechanisms involved.

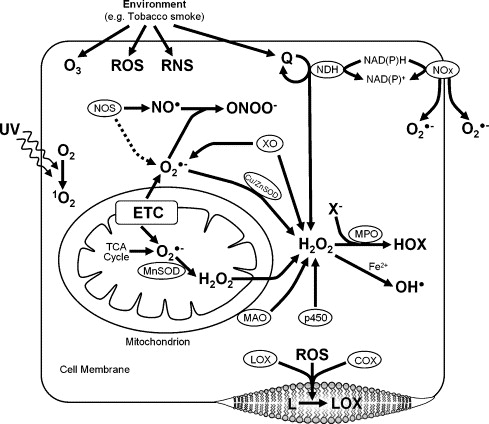
Fig. 1. Cellular sources of reactive oxygen species. Aside from the ETC, TCA (Citric Acid) Cycle, and monoamine oxidase (MAO). ROS can be generated from NADPH oxidases (NOX), UV light, and smoke. Other sources, including the Cu/Zn superoxidative species (Cu/Zn SOD) and nitric oxide synthase (NOS) catalysis of NOradical dot are shown to produce H2O2 and peroxynitrite (ONOOradical dot), respectively. Further, H2O2 can react with metals, such as Fe2+, to generate the hydroxyl radical (radical dotOH), myeloperoxidase (MPO) uses H2O2 and halides (X) to make hypohalous acids (HOX), and quinones (Q) may cyclically react with NAD(P)H dehydrogenases to generate ROS (Sheu).

Figure 1 shows various sources of ROS. This experiment will mainly examine sources of ROS from the first cytochrome complex of the ETC.

“Protocol for Mitochondrial Isolation with Enzymatic Disruption”

Yeast was cultured using 10g of dextrose, 80 ml of water and 1 g of yeast in a 150 mL flask. Four trials were performed using the yeast culture. To each test tube, 0.5mL of the yeast solution and 4 mL of YPD broth was added. 0.50mL of 10μM quercetin was added to three of the test tubes. The fourth test tube served as a control. The test tubes were incubated at 30o C for 72 hours in a water bath. After incubation, the samples were centrifuged for 5 minutes at 3500rpm. The supernatant was decanted and the remaining supernatant was removed via aspiration using a 20μL pipette. The samples were suspended in 2 mL of buffer containing 1.0M sorbital, 0.1M EDTA with 0.1% β-ME and 0.1% lyticase added just before use. The pellet was loosened and mixed with the buffer by flicking the test tube. The samples were incubated in a 30oC water bath with gentle shaking for 25 minutes. The samples were centrifuged for 5 minutes at 3500 rpm. The supernatant was decanted and the remaining supernatant was removed via aspiration using a 20μL pipette. 2mL of ice cold homogenization buffer (0.6M mannitol, .01M Tris-CL, .001M PMSF and 1% BSA) was added to the each pellet. Each sample was vortexed for about 1 minute using a VWR analog vortexer on setting 6. The samples were centrifuged for 5 minutes at 3,500 rpm and the supernatant was discarded. The pellets were suspended in 2mL of homogenization buffer. The samples were mixed via flicking the side of the test tube to ensure mixing of the pellet and buffer. The samples were centrifuged for 5 minutes at 1500 rpm. The supernatant was transferred to a clean centrifuge tube and the pellet was discarded. The supernatant was centrifuged for 15 minutes at 12000 rpm. The supernatant was discarded and the pellet was suspended in 2mL of suspension buffer (0.6 M mannitol and .01 M Tris-Cl). The samples were then used immediately to test mitochondrial function using a YSI 5300A Biological Oxygen Monitor (Qiagen).

“Protocol for Mitochondrial Isolation with Mechanical Breakdown” –Ann Schmitt

Yeast was cultured using 10g of dextrose, 80 ml of water and 1 g of yeast in a 150 mL flask. Four trials were performed using the yeast culture. To each test tube, 0.5mL of the yeast solution and 4 mL of YPD broth was added. 0.50mL of 10μM quercetin was added to three of the test tubes. The fourth test tube served as a control. The test tubes were incubated at 30o C for 72 hours in a water bath. After the incubation, 2mL of the sample was centrifuged for 5 minutes at 3,500rpm. The supernatant was discarded and each pellet was washed in 1.0mL of ice cold DI water. The pellet was then suspended in 1mL of a mixture containing 20mM DTT and 100mM Tris. The sample was centrifuged for 5 minutes at 3,500rpm and the supernatant was discarded. 2mL of ice cold homogenization buffer (0.6M mannitol, .01M Tris-CL, .001M PMSF and 1% BSA) was added to the each pellet. The samples were then stored on ice for five minutes. The samples were homogenized using a 2mL Wheaton glass-teflon homogenizer for 5 strokes. The samples were centrifuged for 5 minutes at 3,500 rpm and the supernatant was discarded. The pellet was suspended in 2mL of homogenization buffer and homogenized using a 2mL Wheaton glass-teflon homogenizer for 5 strokes. The samples were centrifuged for 5 minutes at 1,500 rpm. The supernatant was transferred to a centrifuge tube and the pellet was discarded. The supernatant from each sample was centrifuged for 15 minutes at 12,000rpm. The supernatant was discarded and the pellet was suspended in 2mL of suspension buffer (0.6 M mannitol and .01 M Tris-Cl). The samples were then used immediately to test mitochondrial function using a YSI 5300A Biological Oxygen Monitor (Qiagen).

10 microliters of ADP were injected into each sample being tested under the oxygen monitor after a plateau of oxygen consumption level was observed.

Results

These results of the blank trials used for practice demonstrate the stage IV and stage III oxygen consumption of mitochondrial with the addition of ADP at the end of each plateau. Two distinct curves shuold be seen that separate three plateaus. These trials were simply tests to understand and maintain the technique of the protocol.

References:

Held, Paul. "An Introduction to Reactive Oxygen Species - Measurement of ROS in Cells."*BioTek*. BioTek Instruments, 23 Feb. 2010. Web. 23 July 2013.

Qiagen. (2010) Protocol: Purification of Total RNA from Yeast. *RNeasy Mini Handbook.*

Ross, Edward A., Lilia C. Koo, and James B. Moberly. "Low whole blood and erythrocyte levels of glutathione in hemodialysis and peritoneal dialysis patients." *American journal of kidney diseases* 30.4 (1997): 489-494.

Sheu, Shey-Shing, Dhananjaya Nauduri, and M. W. Anders. "Targeting antioxidants to mitochondria: a new therapeutic direction." *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1762.2 (2006): 256-265.

Sohal, Rajindal S., Hung-Hai Ku, Sanjiv Agarwal, Michael J. Forster, and Harbans Lal. "Oxidative Damage, Mitochondrial Oxidant Generation and Antioxidant Defenses during Aging and in Response to Food Restriction in the Mouse." *Mechanisms of Ageing and Development* 74.1-2 (1994): 121-33. Print.