Identification of Enzymes Causing Redox Cycling, Causing Oxidative Stress and Damaging Mitochondria

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University Honors Thesis

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ABSTRACT

Oxidative stress is thought to contribute to the death of dopamine neurons and the consequential progression of Parkinson’s disease. Redox cycling of quinones, including compounds formed by dopamine oxidation, can lead to oxidative stress and damaging of the mitochondria. Which enzymes catalyze the redox cycling of quinones is unknown, however. Inhibitors of the mitochondrial respiratory chain (cyanide, rotenone) do not inhibit redox cycling. Mitochondrial NAD(P)H:quinone oxidoreductases are likely candidates, but dicumarol, a common inhibitor of such enzymes is not effective. We observed that Cibacron Blue does indeed inhibit NADH-dependent redox cycling to an extent. Our data shows that the rate of oxygen consumption decreased with the addition of Cibacron Blue. Moreover, the changes in the mitochondrial membrane potential, indicated by fluorescence of the rhodamine dye bound to the membrane, are less
significant when the inhibitor was added. Cibacron Blue inhibits a quinone:oxidoreductase enzyme and has important implications for the causes of Parkinson’s disease. Moreover, another inhibitor, mersalyl acid, has also been identified as a potentially more effective blocker to redox cycling, based on the drastic decrease in oxygen consumption upon its addition.

INTRODUCTION
Redox reactions are necessary for all life including humans, as they provide the energy for metabolism. However, free radicals and other chemicals produced from redox (reduction-oxidation) reactions can damage cells. Enzymes in mitochondria, the energy centers of cells, can facilitate these reactions and lead to conditions such as Parkinson’s disease. Damage to cells diminishes ATP production and the use of ATP-sensitive channels within dopaminergic neurons. Damage may also trigger apoptosis (programmed cell death) and disrupt cell functions more generally.

A mitochondrial NAD(P)H:quinone oxidoreductase is the suspected cause of redox cycling of quinones, leading to oxidative stress. Quinones are aromatic compounds, which can cycle between reduced and oxidized forms. They are reduced enzymatically and oxidized by reacting with molecular oxygen. This latter reaction produces reactive oxygen species including superoxide and hydrogen peroxide and thereby creates oxidative stress. Recently, it has been discovered that Cibacron Blue, an inhibitor, can be successful in preventing damaging redox reactions.

Our study focuses on redox cycling by catecholamine oxidation products, which may relate to the molecular causes of Parkinson's disease. The central question of this
research project is to determine which NADH-dependent enzymes are involved in redox cycling of quinones and therefore may cause oxidative stress and ultimately kill cells. This phenomenon may contribute to a number of common and serious disorders including Parkinson’s, Alzheimers and cardiovascular disease. I used inhibitors, including Cibacron blue, dicumarol and chrysin to discriminate among these enzymes. If an inhibitor (e.g., Cibacron Blue) is determined be successful in stopping redox cycling by a particular enzyme and it also protects against damage to the cell, this enzyme would be implicated in causing oxidative stress.

The project is separated into two parts. The first part measures redox cycling of 3-methyl-5-anilino-1,2-benzoquinone (MAQ, a test quinone) by NADH-dependent enzymes using the oxygen electrode. One enzyme, a mitochondrial NAD(P)H:quinone oxidoreductase, is the suspected cause of redox cycling (S. Endo 1326). The hypothesis is that this enzyme is inhibited by Cibacron Blue, but not by dicumarol or chrysin, inhibitors of other NADH-dependent enzymes previously thought to cause the redox cycling.

To measure redox cycling further, diaphorase, a cytoplasmic enzyme used to catalyze the reduction of dyes, or hydrogen acceptors, will be tested for redox cycling of MAQ (R. Li 8846). The hypothesis is that this enzyme is inhibited by dicumarol and chrysin, unlike the mitochondrial NADH-dependent enzyme. The question at hand for this part of the experiment is to determine if Cibacron Blue is also successful in inhibiting cytoplasmic diaphorase as it was successful in inhibiting the mitochondrial NADH-dependent enzyme.
The second part of the research project measures the destruction of the mitochondrial inner membrane potential. There are two major questions in this part of the experiment. Do NADH and MAQ together destroy the membrane potential? And also, does Cibacron Blue protect the membrane potential from being destroyed? The hypothesis here is that when NADH and MAQ are added to mitochondria, redox cycling will occur and damage the mitochondrial membrane. Then, the mitochondrial inner membrane will not be able to support a proton gradient and no membrane potential will be observed when succinate, an electron donator to the electron transport chain, is added. The idea is that Cibacron Blue may be able to prevent this just as it was able to inhibit the affects of the enzymes in the first part of the research project. Dicumarol and chrysin, inhibitors of diaphorase, but not of the mitochondrial enzyme, should not have this effect.

MATERIALS AND METHODS

**Method for using oxygen electrode:** Oxygen concentrations in solutions were measured using an oxygen electrode as described by Li et al. (2007). (G. Li 6978). First, the buffer (four milliliters) was placed in the chamber of a Clark-type oxygen electrode. Next, the enzyme preparation and MAQ were added and the O$_2$ concentration is recorded. After a constant baseline is obtained, the NADH was put in the solution and the rate of O$_2$ consumption is recorded. (The enzyme preparations were obtained from veal brain either as mitochondrial (NADH:quinone oxidoreductase) or cytoplasmic (diaphorase) extracts.
Method for membrane potential using fluorescence spectrometer:

Tetramethylrhodaminemethylester (TMRM) fluorescence was used as an indicator of membrane potential. TMRM is reddish-colored dye. Its binding to the membrane changes with membrane potential, which causes its fluorescence to change. When protons are pumped out of the mitochondria (more negative potential) there is less fluorescence. Fluorescence was measured using the fluorescence spectrometer. Potassium phosphate buffer, mitochondria, and the dye solution were all added together in solution in the fluorescence spectrometer. Succinate, capable of donating electrons to the electron transport chain used for cellular respiration, was then added to the solution. This causes a drop in fluorescence. Membrane potential/fluorescence increases again when oxygen is used up and respiration stops. When MAQ is added, there is much less of a membrane potential response. Cibacron Blue could help counter the negative effect of MAQ, and preliminary tests performed in Dr. Njus’ lab have shown promising results. This method served to establish the correlation between the enzyme and the destructive effect on membrane potential.

RESULTS

Two approaches were used to observe the effects of redox cycling inhibitors. First, the fluorometer was used to measure the mitochondrial membrane potential changes upon the addition of the inhibitor. Changes in membrane potential result when damaging redox cycling occurs. Reactive oxygen species formed from the reactions include hydrogen peroxide and superoxide. These, produced by enzymes using quinones as substrates,
damage the membrane and prevent the respiration-dependent change in membrane potential.

*Effects of NADH and MAQ on mitochondrial membrane potential.* Fluorescence was recorded from a suspension of heart mitochondria with the potential-sensitive dye TMRM and NADH and MAQ as indicated. Succinate was added at t=0 to initiate respiration. The fluorescence drops as proton-pumping changes the membrane potential
 across the mitochondrial inner membrane. This fluorescence returns to the original value at later times when respiration depletes the oxygen in the solution. It is apparent that redox cycling of MAQ in the presence of NADH diminishes the respiration-dependent change in membrane potential.

\[ \text{Cibacron Blue protects against the redox-cycling-dependent loss of membrane potential.} \]

Fluorescence was recorded from a suspension of heart mitochondria with the potential-sensitive dye TMRM and NADH and MAQ as indicated. Succinate was added at \( t=0 \) to initiate respiration. The respiration-dependent change in membrane potential was not diminished by NADH and MAQ when Cibacron Blue was present.
A newly discovered inhibitor, mersalyl acid, has proven to be successful in inhibiting radical-forming enzymes. The success is based on trials performed in which the rate of oxygen consumption decreased drastically upon the addition of mersalyl acid. Mersalyl is an organomercury compound. Its structure is important as it may give insight into how it reacts with enzymes that cause oxidative stress. Mersalyl seems to be even more productive than the previously successful inhibitor, Cibacron Blue.

Comparison between the effects of Cibacron Blue and mersalyl acid in terms of the rate of oxygen consumption (measured by the slope of the line). A smaller slope represents a slower rate of oxygen consumption and hence, less formation of free radicals: this would indicate an inhibitor’s efficacy. While Cibacron Blue is a successful inhibitor as indicated by the previous graphs indicating Cibacron Blue’s effect on mitochondrial membrane potential, according to the oxygen electrode models, mersalyl is more effective. While the slope in the presence of mersalyl acid (indicating the rate of oxygen consumption) remains near zero and constant, the slope with Cibacron Blue drops sharply. In essence, oxygen is being consumed at a faster rate.
Comparison of the rate of oxygen consumption with and without mersalyl acid. After the ten minute mark, oxygen consumption increases rapidly without mersalyl acid (indicated by a drop in the slope of the line). Meanwhile, with mersalyl acid, the slope stays relatively constant and is close to zero. This indicates that very little oxygen, which can presumably be used to form free radicals, is being consumed upon the addition of mersalyl acid.

CONCLUSIONS
Three important conclusions have resulted from this research. First, redox cycling of MAQ in the presence of NADH damages the mitochondrial membrane potential. Second, Cibacron Blue inhibits a specific quinone oxidoreductase enzyme that catalyzes redox cycling that leads to oxidative stress. Finally, mersalyl acid is an effective inhibitor in preventing the redox cycling of quinones. Which specific enzyme is inhibited by mersalyl acid, however, is unknown. The discovery of inhibitors of redox cycling inducing enzymes in dopaminergic neurons may prevent their destruction, and possibly solve the mystery of Parkinson’s disease.
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Literature Cited
