Novel approaches to treat mitochondrial complex-I mediated defects in disease

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ABSTRACT

Dysfunction within complex I (CI) of the mitochondrial electron transport system has been implicated in a number of disease states ranging from cardiovascular diseases to neuro-ophthalmic indications. Herein, we provide three novel approaches to model and study the impacts of injury on the function of CI. Cardiovascular ischemia/reperfusion (I/R) injury has long been recognized as a leading contributor to CI dysfunction. Aside from the physical injury that occurs in the tissue during the ischemic period, the production of high levels of reactive oxygen species (ROS) upon reperfusion, led by reverse electron transport (RET) from CI, causes significant damage to the cell. With over 700,000 people in the US set to experience an ischemic cardiac event annually, the need for a pharmacological intervention is paramount. Unfortunately, current pharmacological approaches to treat I/R related injury are limited and the ones that have shown efficacy have often done so with mixed results. Among the current approaches to treat I/R injury antioxidants have shown some promise to help preserve mitochondrial function and assuage tissue death. The studies described herein have provided new, more physiologically matched, methods for assessing the impact of potential therapeutic interventions in I/R injury. With these methods we evaluated the efficacy of the coenzyme-Q derivative idebenone, a proposed antioxidant. Surprisingly, in both chemically induced models of I/R and I/R in the intact heart, we see no antioxidant-based mechanism for rescue. The mechanistic insight we gained from these models of I/R injury directed us to further examine CI dysfunction in greater detail. Through the use of two cutting edge genetic engineering approaches, CRISPR/Cas9 and Artificial Site-specific RNA Endonucleases (ASRE), we have been able to directly edit the mitochondria to accurately model CI dysfunction in disease. The use of these genetic engineering technologies have provided first in class methods for modeling three unique mitochondrial diseases. The culmination of these projects has provided tremendous insight into the role of CI in disease and have taken a significant step towards elucidating potential therapeutic avenues for targeting decrements in mitochondrial function.

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GENERAL AUDIENCE ABSTRACT

Within the mitochondria, "the powerhouse of the cell," exists a series of five enzyme complexes that produce 90% of the energy for our cells need to function. The largest of these enzymes, complex I (CI), plays an important role in ensuring proper mitochondrial function. Injury to CI contributes to a number of diseases, but surprisingly few options exist to treat complex I. One of the most prevalent forms of CI dysfunction can be seen in ischemia/ reperfusion injury, a form of which is most commonly recognized as a heart attack. Surprisingly, the American Heart Association reports that in the next year over 700,000 people in the US will suffer from an ischemic event. With such a profound impact on the population, the need for new therapeutic developments is extremely high. Some current therapeutic approaches have been shown to be effective at treating cardiac dysfunction, but few address the dysfunction that occurs in the mitochondria. Here we test both a method for modeling these ischemia/reperfusion-based injuries and a potential therapeutic for treating these injuries within the context of CI dysfunction. We further evaluate CI dysfunction by using both established genetic engineering approaches as well as a completely new method to model CI disease. Through the use of two cutting edge genetic engineering approaches, we have been able to directly edit components of the mitochondria to accurately model CI dysfunction in disease. The use of these genetic engineering technologies have provided a first-in-class method for modeling three unique mitochondrial diseases. The culmination of these projects has provided tremendous insight into the role of CI in disease and have taken a significant step towards elucidating potential therapeutic avenues for targeting decrements in mitochondrial function.

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Attribution

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Chapter 1

Introduction

After millions of years of a shared symbiotic relationship, we are only now beginning to fully appreciate the impact the mitochondria have on influencing virtually every aspect of life as we know it. For decades mitochondria have been lauded as the "powerhouse of the cell," and have earned this moniker due to the massive amounts of energy they produce in cells across tissues. However, as we will see the mitochondria are responsible for much more than just energy production. A closer look at this unique system shows a collection of five multimeric protein complexes, encoded by both nuclear DNA and the mitochondria's own genome (mtDNA). The electron transport system coordinates successive, thermodynamically favorable redox reactions that begin with the utilization of carbon sources donated from food intake and culminates in the production of energy in the form of ATP. Along this chain, protons are pumped from within the mitochondrial matrix to the intermembrane space at complexes I, III and IV. The pumping of these protons across the inner mitochondrial membrane establishes an electrochemical gradient that is used at complex V to phosphorylate ADP to ATP. Dysfunction among any of these complexes often leads to severe decrements in energy production, an increase in reactive oxygen species production, and ultimately cell death. Despite the recognized importance of mitochondria, there is a lack of reliable, physiological models for assessing dysfunction among the components of the electron transport system. Here we seek to add to what is currently known about the impacts of mitochondrial function in health and disease through cutting edge approaches that model and evaluate the outcomes of mitochondrial dysfunction and support the role of mitochondria as a vastly underappreciated therapeutic target[1–3].The subsequent chapters will focus on work that has predominantly been accomplished in the heart, an important tissue to study, as mitochondria

make up between 25-30% of cardiac cell mass by volume, and contribute 90% of the ATP required for cellular energy production via oxidative phosphorylation[4,5]. Within the context of the heart, the majority of the work discussed herein is focused on complex I (CI) dysfunction, methods for accurately modeling and assessing this dysfunction, and possible therapeutic interventions to restore CI function.

Recent evidence suggests that cardiovascular pathologies contribute to one in every three deaths in the industrialized world, and the need for new approaches to treat cardiac diseases is at an all-time high [1,2]. Mitochondria are key contributors to the progression of cardiac diseases including heart failure [6–9], genetic cardiomyopathy [10,11] and ischemia/reperfusion (I/R) injury [12–15]. I/R injury is of profound interest because the mechanism of injury is broadly applicable to all tissues and has been considered a neglected therapeutic target[16]. While it is the focus here, I/R injury is not limited to the heart. The ischemic portion of the injury results from a loss of blood flow via an occlusion in the vasculature which causes a severe decline in ATP content as well as oxygen delivery to terminal tissues[17]. Although the ischemic portion of injury severely damages the tissue, the reperfusion phase is arguably as dangerous and perhaps even worse. Among the multi-faceted cellular assaults that the myocardium experiences during ischemia/reperfusion, mitochondrial dysfunction is a well-established contributor. The myriad of bioenergetic impairments include ROS emission that exceeds scavenging capacity [18], mitochondrial supercomplex dissociation [8,19,20], cardiolipin loss [12,20,21], altered mitochondrial dynamics [22,23], and opening of energy-dissipating inner membrane channels/pores [24]. Central to each of these bioenergetic impairments is perhaps the most impacted component of the electron transport system (ETS), complex I (CI). As the largest enzyme complex within the ETS, CI dysfunction has been implicated across a number of cardiac

pathologies including I/R injury [12,14,15,18,25–29]. We believe that I/R injury serves as a powerful model of mitochondrial dysfunction and provides a way to garner novel insights about precisely when and where mitochondrial therapeutics may be needed. Chapter 2 describes a novel method for simultaneously assessing mitochondrial reactive oxygen species production and oxygen consumption. This method helps to circumvent some of the current issues in measuring ROS production [30] by providing a physiological context from which to draw conclusions about specific influences on ROS production. In addition, our approach provides a reliable method against which new therapeutic treatments, targeting ROS production, can be evaluated.

Previous approaches to therapeutically target the mitochondria by mitigating the ROS burden in I/R injury have focused on the use of antioxidants[31–33]. In Chapter 3, we provide a new perspective on the mechanism of action of one of these proposed "antioxidants" using a vertically integrated approach, within the heart, targeting a specific form of ROS production known as Reverse Electron Transfer (RET). Our previous method from Chapter 2 validated the way in which RET is modeled and provides a reliable platform for the evaluation of one of these proposed antioxidants, idebenone, on ROS production following I/R. The surprise in assessing the impact of idebenone with several different approaches for modeling RET induced ROS production after I/R injury, as discussed in Chapter 3, is that we find no discernable evidence of ROS scavenging (as has been proposed in the field). We do, however, show clear evidence that idebenone is acting to bypass the RET-induced deficiencies at CI by stimulating the Q-pool to maintain subsequent redox reactions that can mitigate the loss of bioenergetic function. Despite the strength of the data and the well documented injury to CI post I/R, this physical injury model has its limitations. Specifically, it is a brute force approach to model CI dysfunction. Small genetic mutations in the proteins responsible for CI assembly are centrally involved in mitochondrial

disease progression[34]. Chapter 4 describes our attempts to precisely model mitochondrial disease by specifically targeting subunits within CI by genetic engineering.

A majority of the proteins needed for mitochondria to function, about 99%[35], are encoded by the nucleus. In mammals, mitochondrial DNA encodes 37 genes, the majority of which are required translational machinery (22 tRNAs, 2 ribosomal subunits). Thus, there are only 13 genes that are specific for four of the five complexes of the electron transport system, as complex II is completely nuclear encoded. Of these 13, over half (7) are required to make a functional CI. In Chapters 2 and 3, we focus broadly on CI dysfunction after a physical injury like ischemia/reperfusion. In Chapter 4, we focus very specifically on CI dysfunction that results from direct mutagenesis of 2 of the 45 subunits that comprise CI, engineering phenotypic presentations of mitochondrial disease.

Mitochondrial diseases are one of the most common forms of genetic disease with a prevalence estimated to be as high as 1 in 5,000 [36]. Within the context of the heart, altered mitochondrial function can be seen in as many as 1 in every 500 individuals [8,37]. The dysfunction seen in these types of mitochondrial cardiomyopathy differ from acutely injured mitochondria, discussed previously, in that they are almost exclusively linked to alterations in gene expression from either the nuclear or mitochondrial genome. These alterations in gene expression result in dysfunctional protein assembly within the enzymes of the electron transport system (ETS) that ultimately decrease the ability of the mitochondria to produce ATP. While it is clear that mitochondrial dysfunction plays a key role in the progression of a variety of disease states, no viable treatments currently exist for mitochondrial diseases. This lack of therapeutic treatments is due, in part, to the difficulty in creating experimental models of mitochondrial disease. Targeted disruption of proteins encoded by mitochondrial DNA has been bolstered in recent years by several

promising approaches, yet each of these has their limitations. In Chapter 4, we used Artificial Site-specific RNA Endonucleases (ASREs), a novel mitochondrial RNA engineering platform, and a CRISPR/CAS9 - driven approach to create cell-based models of disease. Due to difficulty in growing primary ventricular myocytes in culture, Human Embryonic Kidney cells and mouse muscle myoblasts were utilized for the studies in Chapter 4. While both cell types are a step removed from a cardiac-specific cell line, the kidney and muscle cells are still highly metabolically active [38], and as such provide a suitable surrogate host for these genetic manipulations.

In the subsequent chapters, we will highlight the utility of our novel approaches to treat complex-I mediated defects in disease. When viewed individually we have provided validation of a model for screening mitochondria-targeted therapeutics focused on decreasing RET-induced ROS production. We have discovered a paradigm-shifting assessment of a known, clinically safe, compound and found it to be cardioprotective in the absence of its proposed antioxidant-based mechanism. Finally, we have demonstrated that we can successfully model CI disease using genetic engineering technologies. Taken together, we provide strong evidence that CI is centrally involved in disease progression and show that the mitochondria are a viable target for future therapeutic development.

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Chapter 2

Simultaneous assessment of mitochondrial oxygen consumption and H_2O_2 production via reverse electron transport: methodological considerations for therapeutic compound screening in cardiac ischemia/reperfusion injury.

Abstract

The deleterious effects of reactive oxygen species (ROS) have been indicated across a number of pathologies ranging from adverse cardiac events to cancer. The transient nature of ROS makes them difficult to manage and even more difficult to therapeutically target. To further complicate matters, the method by which ROS are produced is not uniform across tissues. Herein, we focus on modeling the predominant form of ROS production in the heart during ischemia/reperfusion injury, reverse electron transport (RET). Across several experimental conditions we have shown that a unique and reliable, *in vitro,* method for assessing RET-induced ROS production while simultaneously measuring oxygen consumption can be achieved. Since a number of factors can influence the potential efficacy of a novel therapeutic, we sought to establish comprehensive methods to provide a foundation for evaluating the effects of a compound of interest. When respiration and H_2O_2 production were compared in two mitochondrial subpopulations at two different time points, subsarcolemmal (SSM) mitochondria alone yielded lower rates of respiration (1252 ± 43.0 SSM vs. 1846 ± 107.1 pmol/sec*mg SSM+IFM, $P < 0.05$) and ROS production (31.5 ± 2.5 SSM vs. 41.3 ± 4.4 pmol/sec*mg SSM+IFM, $P <0.05$) than SSM and interfibrillar (IFM) mitochondria combined, respectively. We next evaluated the influence of two well established mitochondrial assay buffers to determine what impact, if any, buffer compositions may have on the sensitivity of a succinate-induced ROS assay. Both buffers evaluated showed no significant difference at either time point for any measure of respiration (1689 ± 113 MiR05 vs 1564 ± 42 pmol/sec*mg Buffer Z) or H_2O_2 production (33.0 ± 0.5 MiR05 vs 37.8

± 3.9 pmol/sec*mg Buffer Z). While temperature is known to impact respiratory kinetics, we observed no difference for parameters measured under either of the conditions, on ice or at 37°C. Mitochondria incubated at 37°C in the absence of substrates did display a slight depolarization that lead to a significant decrement in total RET induced ROS production; however, we find these conditions to be non-physiological. To further evaluate our proposed model as a valid screening platform, we evaluated two substrates known to impact the electron transport system in different ways. The complex I inhibitor rotenone corroborated previous studies showing that succinate dependent respiration was unaffected (1342 ± 36.4 control vs. 1262 ± 46.4 pmol/sec*mg rotenone), but RET induced ROS production was significantly diminished (30.2 ± 1.3 control vs. 7.6 ± 0.2 pmol/sec*mg rotenone, $P<0.05$). While previous studies have shown that the coenzyme Q analog, idebenone, behaves as an anti-oxidant, we observed a reduction in both respiration and ROS production in our model of RET. Despite this reduction, no significant difference between the vehicle control and idebenone was obtained when rates of H_2O_2 production where normalized to rates of oxygen consumption (2.5 ± 0.1 vs 2.5 ± 0.1 % H_2O_2/O). When taken in totality, evaluation of the assay's performance across a number of experimental conditions provided a clear, simple, real time assay for investigating the impact of compounds of interest on both RET-induced ROS production and mitochondrial bioenergetics.

Introduction

Within the heart, reactive oxygen species (ROS) production and associated redox signaling are controlled by a myriad of factors and are essential to preserving homeostasis and vitality. ROS are generated via the one electron reduction of freely available molecular oxygen producing a superoxide anion ($O_2^{\bullet-}$) which can dismutate into H_2O_2 (peroxide) or behave as free radicals. At low concentrations, ROS evoke cellular adaptations that support cell survival, but higher, toxic levels of ROS induce cellular damage, aberrant bioenergetics, and cell death [1,2].

Although multiple cellular components can contribute to ROS production[3], the mitochondria are a major physiological producer. ROS are predominantly generated via two locations in the mitochondrial electron transport system (ETS); NADH ubiquinone oxidoreductase (complex I) and the ubiquinol-*cytochrome c* oxidoreductase (complex III). Here, we focus specifically on complex I-mediated ROS production and focus on the creation of an *in vitro* model of RET production driven by ischemia/reperfusion (I/R) injury. The main mechanism of superoxide production in complex I occurs during I/R through Reverse Electron Transfer (RET)[4,5]. Briefly, the citric acid cycle intermediate, succinate, accumulates during ischemia and is rapidly oxidized to drive forward flux through the electron transport chain during the first several minutes of reperfusion[4]. This rapid oxidation of accumulated succinate on reperfusion contributes to an over reduced Quinone-pool, an increased $NADH/NAD^+$ ratio and a low ATP production rate. These changes lead to an alteration in the mitochondrial membrane potential that facilitates a "reversed" flow of electrons back to complex I[4]. RET-induced ROS production is of significant interest in the study of metabolic pathologies as the phenomena has been associated with a number of additional disease states, outside the context of the heart, ranging from chronic neurological indications to cancer[6]. While our primary focus among these indications are on

those that are cardio-metabolic in nature the development of this assay will provide a novel method for evaluating therapeutic compounds across indications.

Since ROS signaling and the management of ROS production have been identified as viable targets for the treatment of cardiovascular diseases[7], a number of assays that assess levels of ROS *in vitro* have been developed[8]. Many of these methods, including the approach described herein, measure the amount of H_2O_2 present in solution with fluorometric probes like Amplex® UltraRed. Current high throughput assays, however, still have difficulty measuring superoxide production *in vivo* as the reliability of probes like dichlorofluorescein (DCF) and Mito-SOX have been questioned in recent years[9]. Additionally, it has been shown that extrapolations from *in vitro* data should be interpreted cautiously as the effects of inhibitors on forward flux through the electron transport system (ETS) and superoxide production *in vitro* have been difficult to reconcile[10]. Here we provide a comprehensive method that narrows this gap by simultaneously measuring respirometry (oxygen consumption driven by forward electron flow) and the fluorometric measurement of succinate-induced ROS production in a highly specific, quantitative, and reproducible manner. Across a range of experimental conditions, buffers, and temperatures we provide a detailed assessment of the simultaneous measurement of respiration and RET-induced ROS production from isolated cardiac mitochondria with an OROBOROS Oxygraph-2K[11]. In addition, we assess the impact of rotenone, a well-established complex I inhibitor and idebenone, a proposed antioxidant, each with their own unique mechanisms of action on RET-induced ROS production as a validation of our model. This assay provides an attractive alternative to existing technologies and shows significant promise for thoroughly ascertaining the effects of experimental conditions and novel compounds on mitochondrial ROS production and respiration.

Methods

The isolation of cardiac mitochondria

Mitochondria were isolated from cardiac tissues as previously described[12]. Cardiac tissues were homogenized on ice in 1:10 w/v of the medium containing 180 mM KCl, 10 mM TrisHCl, and 1 mM EGTA (pH 7.7 at 4˚C) using a Teflon glass homogenizer. To isolate both subsarcolemmal (SSM) and intermyofibrillar (IFM) mitochondria, the homogenate was incubated with subtilisin A (0.5 mg/ml) on ice for 5 min. The homogenate was centrifuged at 750 g for 5 min at 4˚C and the obtained supernatant was centrifuged at 6800 g for 10 min at 4˚C. The obtained mitochondrial pellet was washed once (10 min at 6800 g) and resuspended in the buffer containing 180 mM KCl, 20 mM TrisHCl, pH 7.2 at 4˚C.

High-resolution respirometry with simultaneous H_2O_2 production measurement

To determine mitochondrial function, high-resolution respirometry with simultaneous fluorometry was performed using an Oxygraph-2k (O2k; OROBOROS INSTRUMENTS, Austria) with an O2k-Fluo-Module. All experiments were performed at 37 °C. The measurements were performed in MiR05 (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1, and 0.1% BSA essentially fatty acid free) or in Buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.1, and 0.05% BSA essentially fatty acid free). The medium was reoxygenated when total oxygen concentration fell below 80 µM.

RET was induced by the addition of succinate (10 mM). H_2O_2 flux (ROS flux) was measured simultaneously with respirometry in the O2k-Fluorometer using the H_2O_2-sensitive probe Amplex® Red (AmR) as previously described[11]. 10 µM AmR, 1 U/mL horse radish peroxidase (HRP) and 5 U/mL superoxide dismutase (SOD) were added to the chamber. The HRP catalyzed

AmR reaction with H_2O_2 produces a fluorescent product, similar to resorufin, the levels of which are collected by the O2k-Fluo-Module. Calibrations were performed with H_2O_2 in 0.1 μM steps. For assessment of the effects of temperature a subset of mitochondria were pre-incubated on ice or at 37°C in O2k for 15 min before the assay. For idebenone and rotenone analysis, idebenone (1μM), rotenone (100nM), or a vehicle control (DMSO) were incubated on ice prior to being added to the chamber before addition of mitochondria. Substrate injections for extended respiration studies were: Mitochondria, succinate (10mM), H2O2 (0.1μM), ADP (5mM), pyruvate (5mM), malate (2mM), carboxyatractyloside (Catr) (5μM), and an uncoupler (U), CCCP (0.5μM), volumes are final concentrations.

Data analysis

ROS flux was corrected for background (AmR slope before addition of mitochondria). H_2O_2/O flux ratio [%] was calculated as H_2O_2 flux/(0.5 O_2 flux). All data are expressed as the mean ± standard error of the mean (SEM). For statistical analysis, Student's t-test were used. P values less than 0.05 were considered statistically significant. Statistical calculations were performed using Prism 5.03 software (GraphPad, San Diego, California)

Results and Discussion

A number of methods for measuring ROS production currently exist and have been reviewed in great detail elsewhere [8,9]. Although it is difficult to directly obtain precise quantities of superoxide production due to its extremely short half-life in biological systems, the use of H_2O_2 as a corollary reporter of superoxide generation as measured by Amplex ® Red has been widely accepted[8]. While most models, including this one, rely on a fluorescence-based approach to

measure levels of ROS the novelty of our method is the combination of ROS measurements with the simultaneous collection of oxygen consumption via an Oroboros Oxygraph-2k high resolution respirometer. The major draw for studying ROS production in the Oroboros system is that the platform eliminates ambient light from the chamber, a critical component to control since Amplex® Red has been shown to be highly sensitive to ambient light [13]. Here we utilize the tightly correlated 1:1 stoichiometry of the Amplex® Red system, across experimental conditions, to assess the potential for this assay to become a reliable platform against which novel cardioprotective compounds can be evaluated.

Comparison of cardiac mitochondrial subpopulations

There are two mitochondrial subpopulations in the heart, subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria, that differ in sensitivity to metabolic challenge and respiratory capacities[14–17]. We first assessed if there was a difference in mitochondrial respiration and ROS production between subpopulations during RET conditions. As shown in Fig. 1, after succinate addition the respiration rates as well as H_2O_2 production rates were significantly higher in the mitochondrial preparation containing both mitochondrial subpopulations (SSM+IFM) compared to only SSM fraction at both time points. In order to set the context of ROS production in a more physiological setting we normalized the H_2O_2 production rate to the oxygen consumption rate, as described above. This normalization considers any influence that changes in the rate of respiration may have on over all H_2O_2 production. More simply, if rates of respiration are higher it is possible that rates of H_2O_2 to production will also increase due to the dependence of one on the other[18]. Since we observed no difference in the normalized H_2O_2/O ratio between preparations (Fig.1D), the higher rate of H_2O_2 production observed in SSM+IFM preparations was likely due to higher

O_2 consumption as a result of increased total mitochondrial content as opposed to any type of endogenous increase in total ROS production. These normalized ROS production values corroborate previously reported measurements using electron paramagnetic resonance showing that there was no difference between IFM and SSM in rates of total superoxide levels in RET conditions[14]. Since the procedure of isolation of SSM is simpler and less time consuming than isolation of IFM, and no difference in normalized RET-induced ROS production is observed, the SSM isolations were selected as are preferential preparation for the RET assay for drug compound screening.

Comparison of respiration buffers

In order to assess the impact of different buffers on ROS production we compared two of the most commonly used buffers in cardiac and skeletal muscle mitochondrial studies in the O2k, sucrose-based MiR05 and K-MES based Buffer Z[19–24]. Since Amplex Red sensitivity has been proposed to differ between buffers[25], to ensure comparable sensitivity range for both buffers the measurements were performed using different signal polarization voltage (MiR05-200 mV vs Buffer Z-100 mV). As shown in Figure 2, there is no significant difference in respiration or H_2O_2 production rate between buffers. Thus, both MiR05 and Buffer Z are suitable for the RET assay.

Comparison of pre-incubation conditions

Pre-incubation with a compound of interest is a typical step in screening assays and the conditions under which a compound is incubated plays a significant role in assessing its efficacy. In the present study we assessed the impact of pre-incubation of the mitochondria on ice vs 37°C in an O2k chamber. Interestingly, we noticed that pre-incubation at 37°C in the O2k chamber

moderately slows the transition to maximal respiration rate as well as H_2O_2 production, however neither significantly change overall rates (Fig. 3A-D). Surprisingly, after pre-incubation at 37°C mitochondria produced 22% less H_2O_2 over a 5 min period (Fig.3C) in the absence of any therapeutic intervention. Such difference in measurement pattern could be explained by fact that during 15 min of pre-incubation at 37°C in O2k chamber in the absence of exogenous substrates isolated mitochondria may become depolarized (Fig.3E&F) as suggested by Ross and colleagues[26]. This potential depolarization induced by mild uncoupling could contribute to the decrease in RET by altering the membrane potential as a possible survival mechanism[27]. This data suggests that caution should be used in determining the conditions under which incubation with a compound occurs as potential depolarization of mitochondria could mask the tested compounds effects on mitochondrial respiration and ROS production.

An extended measurement protocol for the evaluation of compound effects on the ETS

It has been shown that compounds known to directly affect the ETS, like complex I inhibitors and/or uncouplers reduce RET-induced ROS production [5,28–31]. To evaluate the effects of tested compounds on the ETS, we propose following an extended RET assay protocol (Fig. 4). After 10 min of incubation with succinate, ADP is added at a saturating concentration. The addition of ADP provides two unique options that must be considered when evaluating new compound efficacy. While endogenous rates of ATP production in a RET induced ROS producing environment would be low, as there is a delay in the regeneration of ADP from AMP[4], the hyperpolarization of the mitochondrial membrane under these conditions could allow for usable proton re-entry through the ATP-synthase. This re-entry is possible if the mitochondrial membrane is less damaged (more tightly coupled) and begins to diminish as membrane potential becomes

less negative. Therefore, stimulation of respiration after addition of ADP may indicate a conservation of mitochondrial coupling under RET conditions. Minimal changes in the rates of respiration after addition of ADP, however, could indicate a damaged mitochondrial membrane as usable proton re-entry through the ATP-synthase may be overshadowed by the amount of leak respiration attributed to a "leaky" membrane.

In this extended model, in the absence of any therapeutic compound, the addition of complex I substrates, pyruvate and malate, causes a pronounced increase in respiration rate, by removing the inhibitory oxaloacetate and restoring CI&II-linked citric acid cycle activity. If no stimulation after addition of complex I substrates is observed, the tested compound could be inhibiting complex I [11]. Taking into account the special considerations with ADP addition discussed above, in our hands, the measurement of respiration rates after addition of the ANT inhibitor (carboxyatractyloside) and an uncoupler at optimum concentration shows coupling and an apparent excess ETS capacity that provides additional data points for potential compound evaluation. This extended protocol provides a powerful tool for additional evaluation of therapeutic compound efficacy while also allowing for the collection of RET data.

The effects of rotenone and idebenone on succinate induced RET

Although a number of recent studies have evaluated the efficacy of novel therapeutic interventions on the mitochondria during I/R injury[32–34], few have combined the simultaneous quantification of oxygen consumption to provide a more physiological assessment of a compounds efficacy. As proof of concept for our model of RET induced ROS production to be a viable method against which to screen compounds, we set out to evaluate two known CI targeted compounds.

Rotenone, a potent inhibitor of CI has been proposed to decrease injury to the myocardium after an ischemia/reperfusion injury[35]. In addition, other studies have demonstrated that the inhibition of complex I during the onset of reperfusion preserves mitochondrial integrity and decreases myocardial infarct size[36–38]. Thus, the first step was to investigate the effect of rotenone in the proposed RET assay. As seen in Figure 6B, the pre-incubation of isolated mitochondria with rotenone (100 nM) induced a significant decrease in rates of H_2O_2 production. If rotenone binds to the ubiquinol binding site of CI as has been proposed[39] it is possible this inhibitory binding helps prevent the Q-pool from dumping electrons back on CI and as a result reduces the total amount of ROS production via a RET based mechanism[6,40]. As expected, the inhibition of complex I by rotenone did not change oxygen consumption rates downstream of the inhibition of CI as evidenced by the absence of response to CI specific substrates (pyruvate and malate) delivered after ADP stimulation (Fig.5 A&E). The treatment of mitochondria with 100nM rotenone corroborates earlier work suggesting a decrement in H_2O_2 production and by association a decrement in H_2O_2/O ratio as well (Fig. 5 C&D).

Recently, it has been shown that the coenzyme Q (CoQ)-pool significantly contributes to ROS production via RET[41]. Moreover, analogues of CoQ, MitoQ and SkQ, have been shown to decrease ROS production and protect the heart against ischemia-reperfusion damage[32,42,43]. One potential mechanism for achieving this cardioprotection could come from the ability of short chain quinone molecules to bypass CI deficiencies[44]. In this model, we studied the effects of idebenone, a short chain CoQ derivative, that has been evaluated in a number of clinical trials and has been shown to be safe in humans[45,46]. Here we hypothesized that if rotenone inhibits CI to decrease RET perhaps bypassing CI deficiency to stimulate downstream bioenergetic flux and relieve the succinate induced mechanism that drives RET could also reduce total ROS production.

To our surprise, the treatment of isolated mitochondria with 1μM idebenone decreased *both* respiration and H_2O_2 production rate (Fig.6A-B). Despite significantly lower rates of respiration and H_2O_2 production, the normalization of idebenone treated mitochondria yielded no difference between idebenone treatment and the vehicle control (Fig.6C). Upon further examination, when the RET respiration assay was extended for longer than 10 minutes we demonstrated that idebenone treatment does not inhibit the ETS (Fig.6D).

Conclusions

Taken together our data support the use of a succinate driven RET based model of ROS production to evaluate compound efficacy. Through the evaluation of a number of conditions we have validated that our model is accurate and reproducible, when coupled with simultaneous collection of oxygen consumption, to yield a reliable answer for potential compound screening. Within the context of the heart and I/R injury our results demonstrate that compounds with cardioprotective potential may reduce total H_2O_2 production, but should be normalized via the H_2O_2/O ratio to ascertain their direct mechanism. The conflicting results we obtained with idebenone treatment warrants further investigation to better understand the role of idebenone on cardioprotection and the potential of transient CI inhibition during I/R to reduce RET during reperfusion. We believe this model provides a way to make these future studies possible. Additionally, this RET assay has the potential to be combined with an extended respiration measurement by addition of other substrates, inhibitors and uncouplers to further assess potential compound interactions on the ETS. Collectively, our data support the use of this combined approach to accurately assess RET and should be used as a screening model not only for cardioprotective compounds but for all tissues in which reducing RET is therapeutically beneficial.

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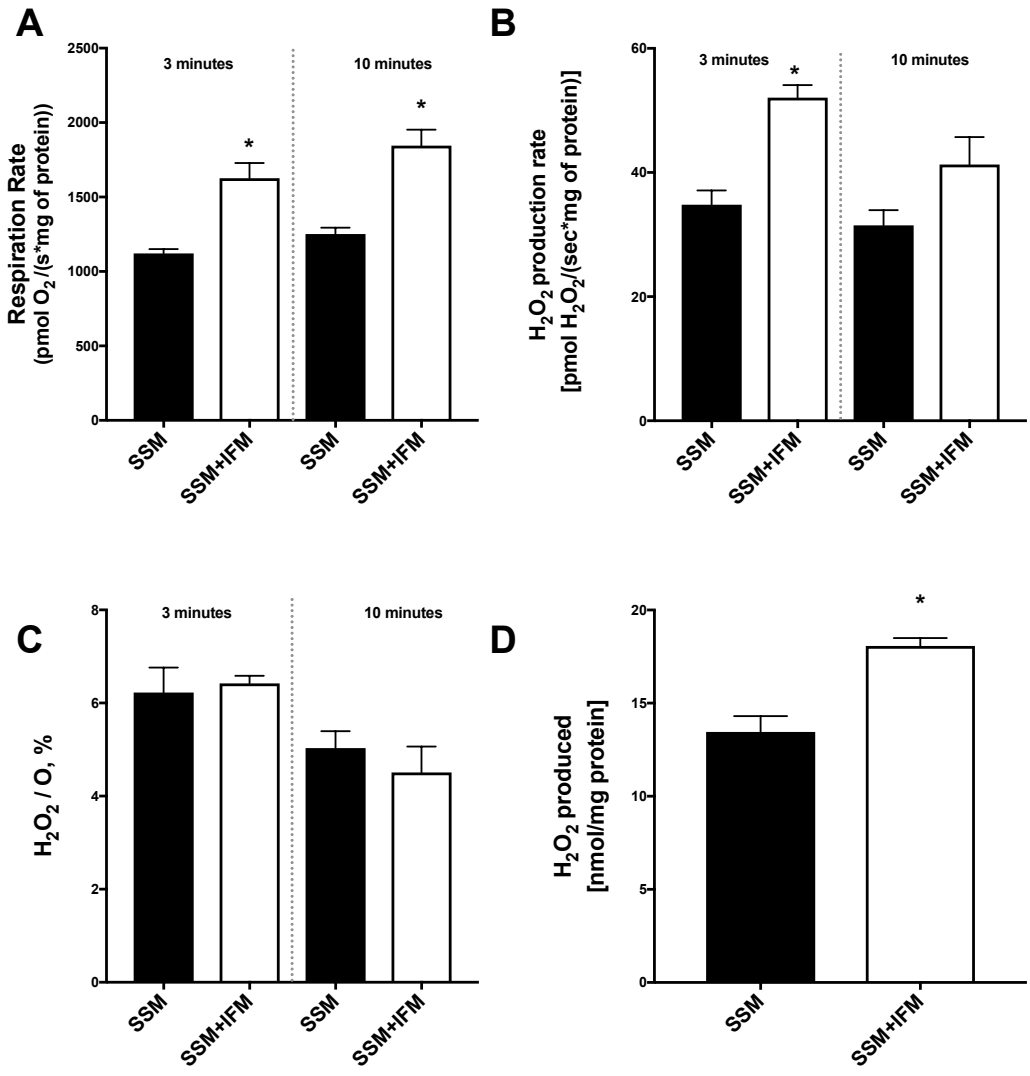


Figure 1. Mitochondrial function from different populations, subsarcolemmal (SSM) and intermyofibrillar (IFM) mitochondria. Rates of respiration (A), early (3 min) H_2O_2 production (B), and H_2O_2 produced during 5 min (D) yielded significant differences between populations. H_2O_2/O flux ratio (C) did not support this difference. The results are presented as the mean ± SEM of 3 measurements. n=3 for both conditions. *, $P < 0.05$ compared to control. Values are ± SEM.

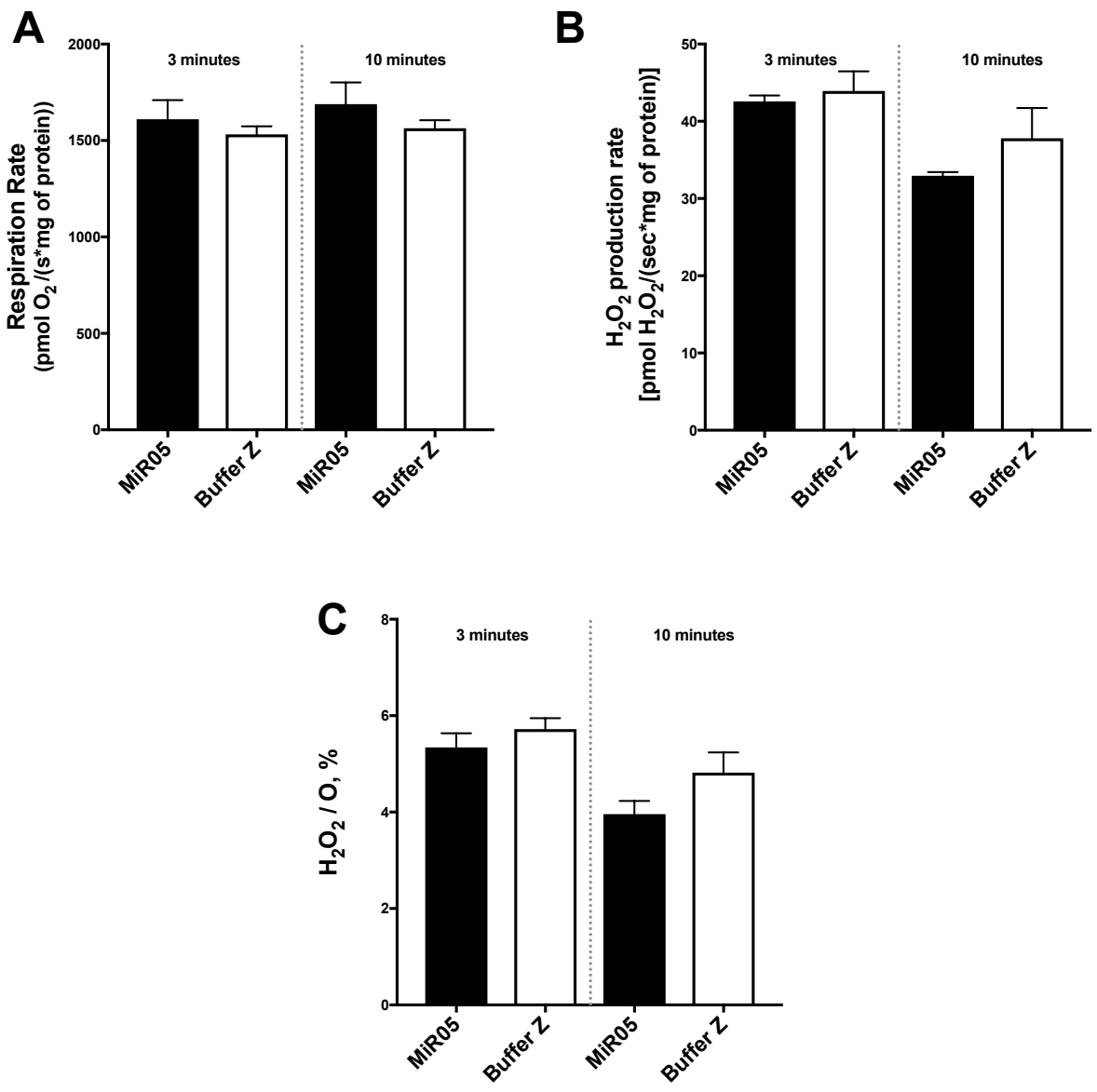
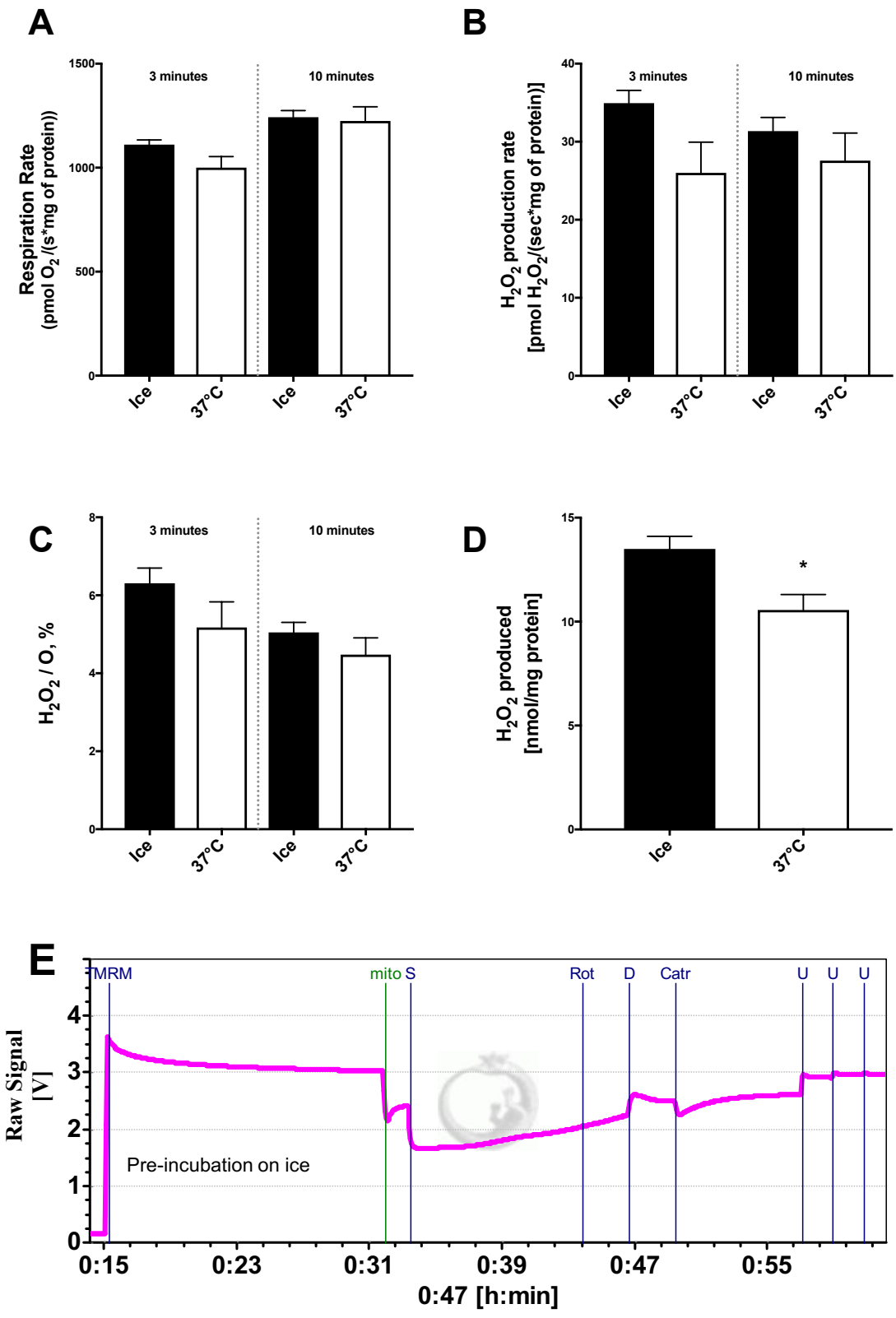


Figure 2. Mitochondrial function in different buffer solutions is unchanged. Rates of respiration (A), H_2O_2 production (B), and H_2O_2/O flux ratio (C) show no difference between the two most commonly used mitochondrial assay buffers. The experiments were performed in a similar sensitivity range (polarization voltage for MiR05-200 mV; for Buffer Z-100 mV). n=4 for both buffers. *, $P < 0.05$ compared to control. Values are ± SEM.


A
Respiration Rate
(pmol O2/(s*mg of protein))
1500
1000
500
0
3 minutes
10 minutes
Ice
37°C
Ice
37°C
B
H2O2 production rate
[pmol H2O2/(sec*mg of protein)]
40
30
20
10
0
3 minutes
10 minutes
Ice
37°C
Ice
37°C
C
H2O2 / O, %
8
6
4
2
0
3 minutes
10 minutes
Ice
37°C
Ice
37°C
D
H2O2 produced
[nmol/mg protein]
15
10
5
0
*
Ice
37°C
E
Raw Signal
[V]
TMRM
mito S
Rot
D
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Pre-incubation on ice
4
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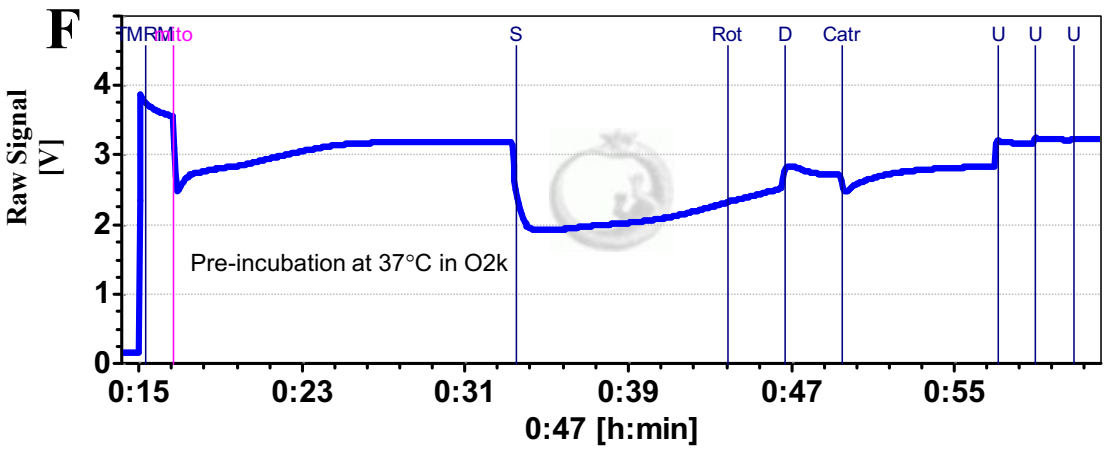

Figure 3. The effect of pre-incubation on mitochondrial function in RET protocol: respiration rate (A); H_2O_2 production rate (B); H_2O_2 produced during 5 min (C); H_2O_2/O flux ratio (D). The representative traces of mitochondrial membrane potential measurements (E). n=4 for ice and n=3 for 37°C. Mito-mitochondrial addition, S-succinate, Rot-Rotenone, D-ADP, Catr-carboxyatractyloside, U-uncoupler. *, P <0.05 compared to control. Values are ± SEM.

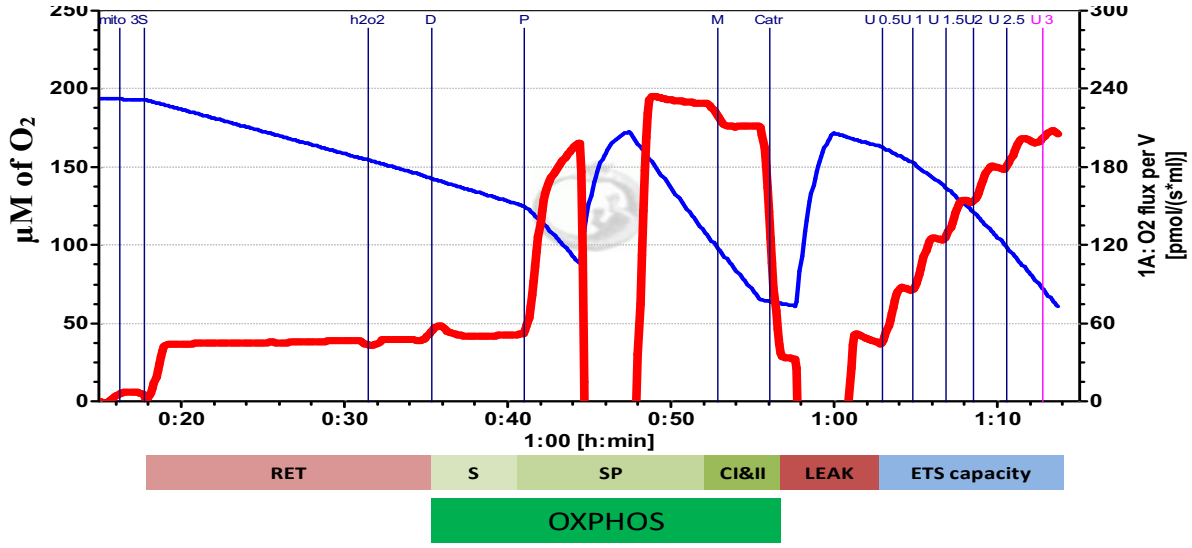

Figure 4. Representative trace of mitochondrial respiration measurement of extended RET assay to determine ETS function. Addition of ADP (D) induces oxidative phosphorylation (OXPHOS). The subsequent addition of pyruvate (P) causes a pronounced increase of respiratory OXPHOS capacity by removing the inhibitory oxaloacetate and restoring CI&II-linked citric acid cycle activity. Addition of malate (M) slightly inhibits mitochondrial respiration and induces CI&II-linked OXPHOS respiratory states. To determine LEAK state respiration and coupling of ETS, the carboxyatractyloside (Catr), ANT inhibitor, was added. Afterwards, uncoupler (U), CCCP, was titrated to determine apparent ETS excess capacity. The blue line represents total O_2 concentration (left y-axis) while the red line represents the flux or change in total O_2 (right y-axis).

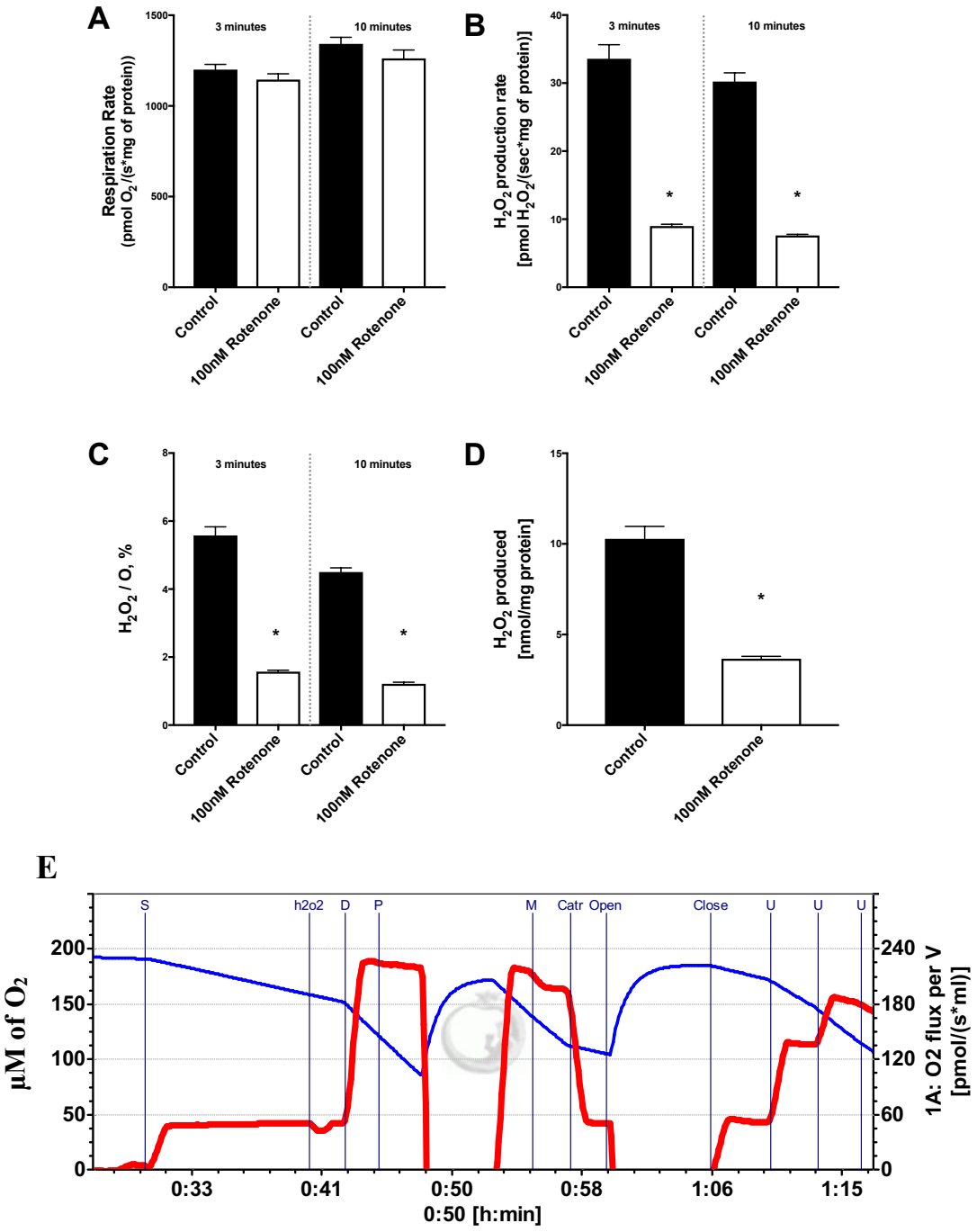

Figure 5. The effect of rotenone on mitochondrial function in an extended RET protocol. Respiration rates were unchanged after rotenone treatment (A). H_2O_2 production rate (B), H_2O_2/O flux ratio (C), and total production decreased after treatment. The representative trace of

mitochondrial respiration during an extended RET assay after treatment with rotenone (E). n=5 for both conditions. *, $P < 0.05$ compared to control. Values are ± SEM.

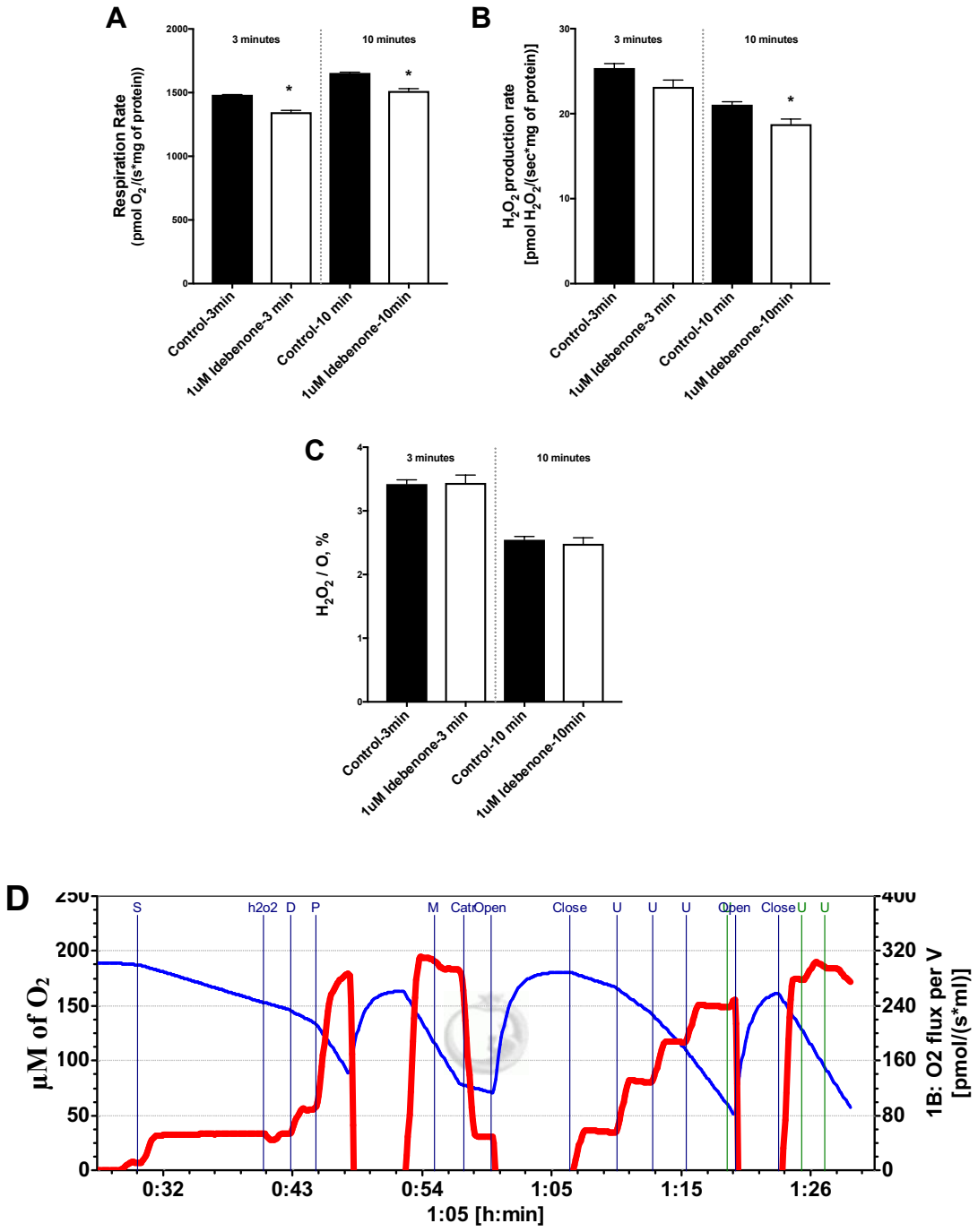

Figure 6. The effect of idebenone on mitochondrial function in an extended RET protocol. Rates of respiration (A), and H_2O_2 production (B) were markedly diminished after idebenone treatment. H_2O_2/O flux ratio (C) however was unaffected. The representative trace of mitochondrial respiration measurement during an extended RET assay after mitochondria treatment with

idebenone (D) shows no inhibition of respiration. n=3 for both conditions. *, $P < 0.05$ compared to control. Values are ± SEM.

Chapter 3

Cardioprotective effects of idebenone do not involve ROS scavenging: evidence for mitochondrial complex I bypass in ischemia/reperfusion injury

***A version of this paper is currently under review with the Journal of Molecular and Cellular Cardiology**

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Abstract

Novel therapeutic strategies to treat mitochondrial deficiencies in acute coronary syndromes are needed. Complex I of the mitochondrial electron transport system is one of the most impaired complexes following ischemia/reperfusion (I/R) injury. This disruption contributes to aberrant electron transport, diminished bioenergetics, an altered redox environment, and mitochondrial damage involved in tissue injury. In this study, we determined the cardiac and mitochondrial effects of idebenone, a benzoquinone currently in several clinical trials with purported 'antioxidant' effects. We employed complementary models of ischemia-reperfusion injury in perfused hearts, permeabilized cardiac fibers, isolated mitochondria, and in cells to elucidate idebenone's cardioprotective mechanism(s). In *ex vivo* whole hearts, infarct size was markedly reduced with post-ischemic idebenone treatment (25 ± 5 % area at risk, AAR) compared to controls (56 ± 6 % AAR, $P<0.05$). Several parameters of hemodynamic function were also significantly improved after idebenone treatment. Parallel studies of anoxia-reoxygenation were conducted

using isolated mitochondria and permeabilized ventricular fibers. In isolated mitochondria, we simultaneously monitored respiration and ROS emission. Idebenone treatment modestly elevated succinate-derived H_2O_2 production when compared to vehicle control (1.34 ± 0.05 vs 1.21 ± 0.05 %, H_2O_2/O_2 respectively, $P<0.05$). Isolated mitochondria subjected to anoxia/reoxygenation demonstrated higher rates of respiration with idebenone treatment (2360 ± 69 pmol/sec*mg) versus vehicle control (1995 ± 101 pmol/sec*mg). Permeabilized cardiac fibers produced high rates of H_2O_2 after anoxia-reoxygenation (0.25 ± 0.04 % H_2O_2/JO_2 compared to 0.12 ± 0.01 % in normoxic fibers, $P <0.05$), with idebenone showing no discernable attenuation on H_2O_2 production (0.26 ± 0.04 % H_2O_2/JO_2). These insights were further investigated with studies in mitochondria isolated from reperfused ventricle. The profound decrease in complex-I dependent respiration after ischemia-reperfusion (701 ± 59 pmolO_2/sec*mg compared to 1816 ± 105 pmol O_2/sec*mg) in normoxic mitochondria was dramatically attenuated with idebenone treatment (994 ± 76 vs pmol O_2/sec*mg, $P<0.05$), supporting a mechanistic role involving complex I. Finally, the effects of idebenone were determined using permeabilized cell models with chemical inhibition of complex I. ADP-dependent oxidative phosphorylation capacity was significantly higher in complex-I inhibited cells treated acutely with idebenone (89.0 ± 4.2 pmol/sec*million cells versus 70.1± 8.2 pmol/sec*million cells in untreated cells). Taken together, these data indicate that the cardioprotective effects of idebenone treatment do not involve ROS-scavenging but appear to involve augmentation of the quinone pool, thus providing reducing equivalents downstream of complex I. As this compound is already in clinical trials for other indications, it may provide a safe and useful approach to mitigate ischemia-reperfusion injury in patients.

Keywords

cardioprotection; idebenone; mitochondria; reactive oxygen species; ischemia; reperfusion

Introduction

Cardiovascular pathologies contribute to one in every three deaths in the industrialized world, and the need for new approaches to treat cardiac diseases is at an all-time high [1,2]. Mitochondria are key contributors to the progression of cardiac diseases including heart failure [3–6], genetic cardiomyopathy [7,8] and ischemia/reperfusion (I/R) injury [9–12]. Given the clear role that impaired mitochondrial function plays across the spectrum of cardiac pathologies, there is enormous opportunity to improve patient outcomes using novel therapeutic approaches.

Among the multi-faceted cellular assaults that the myocardium experiences during ischemia/reperfusion, mitochondrial dysfunction is a well-established contributor. The myriad of bioenergetic impairments include ROS emission that exceeds scavenging capacity [13], mitochondrial supercomplex dissociation [5,14,15], cardiolipin loss [9,15,16], altered mitochondrial dynamics [17,18], and opening of energy-dissipating inner membrane channels/pores [19]. Central to each of these bioenergetic impairments is perhaps the most impacted component, complex I (CI) of the electron transport system (ETS). As the largest enzyme complex within the ETS, CI dysfunction has been implicated across a number of cardiac pathologies including I/R injury [9,11–13,20–24].

Several pathways of I/R injury have been the target of therapeutic interventions. Commonly employed approaches include the use of proposed "ROS scavengers" to restore function to damaged mitochondria [21,25–33]. Despite the theoretical potential of ROS-scavenging molecules to reduce oxidative stress and decrease disease burdens, ROS-scavenging approaches have not translated well in clinical trials across indications [34–36]. The reasons for

lack of translation has been described elsewhere [37]. A primary contributor is that most pre-clinical compounds do not progress to clinical use in humans. To advance clinical treatments for reperfusion injury, we repurposed a compound already shown to be safe in humans. Accordingly, we tested the hypothesis that the benzoquinone idebenone would protect the myocardium when administered at the onset of reperfusion.

Idebenone's therapeutic potential for protecting the myocardium is bolstered by studies where it has been shown to be safe and well tolerated in humans [38–40]. Despite a favorable safety profile, idebenone's mechanism of action is not clear. While some have demonstrated idebenone to be efficacious in reducing ROS production [41,42], we speculate that the protection observed across injury paradigms with idebenone stems from its intrinsic ability to act as a redox cycler [43] and not as an anti-oxidant. More simply, because idebenone can both accept and donate electrons, it has the capacity to facilitate subsequent redox reactions downstream of ETS deficiencies, thereby stimulating respiration [29,42,44,45]. In this study we used idebenone across parallel models to determine if it is cardioprotective, and attempted to elucidate the mechanism by which this protection is achieved.

Materials and Methods

All reagents were obtained from Sigma-Aldrich unless otherwise indicated. All animal research received prior approval from the Institutional Animal Care and Use Committees from our respective institutions (Virginia Tech and the Latvian Institute for Organic Synthesis). Animals were housed in a light- and temperature-controlled environment and received food and water ad libitum.

Perfused Heart Studies

For whole heart studies, male Sprague Dawley rat hearts were excised, instrumented, and subjected to ischemia/reperfusion on one of four parallel Langendorff apparatus per our established protocols [19,46,47]. After 20 minutes of ischemia, a subset of hearts received 1μM idebenone (n=5, 2 hearts were excluded due to perfusion issues occurring during the protocol) or DMSO control (n=8) at the onset of reperfusion, with treatment continuing until the end of the protocol (for 120 minutes). Hemodynamic parameters were digitized and continuously collected throughout and infarct size was quantified at the end of the protocol as previously described [46].

Cardiac Mitochondrial Function

Mitochondria were isolated from rat heart using our established methods [46]. Respiratory control ratios (RCRs) were measured using glutamate/malate (10mM/2mM respectively) and ADP (5mM) substrate (37ºC) for each isolation by calculating State 3/State 4 rates of respiration [48,49]. If RCRs from normoxic mitochondria were less than 6 the preparation was not used. Mitochondrial function was determined using previously established protocols [50,51]. Briefly, we simultaneously measured oxygen consumption and H_2O_2 production via high-resolution respirometry in the Virginia Tech Metabolism Core using one of five Oxygraph-2ks ("O2Ks"; Oroboros Instruments, Austria). All experiments were performed at 37ºC in Buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM creatine, 1 mM EGTA, 0.05% BSA, and pH 7.1) with 10 μM Amplex Red (AmR; ThermoFisher Scientific), 4 U/ml Horseradish peroxidase (HRP), and 30 U/ml Superoxide Dismutase added for detection of reactive oxygen

species (ROS) production. H_2O_2 calibrations were performed using 0.1 μM titrations to generate a standard curve. Mitochondria from normoxic hearts were used for all experiments unless otherwise indicated below.

Reverse Electron Transfer (RET) Assay

A subset of mitochondria were allowed to incubate in the O2k chamber for 15 minutes with 1μM idebenone (n=9), 10μM idebenone (n=8), or a vehicle control (n=16 pooled, one control per condition on each O2k) prior to RET assessment. RET was induced by the addition of 10mM succinate to isolated mitochondria in State 4 conditions and ROS production was monitored for the duration of the protocol. Levels of ROS production were analyzed by integrating the area under the curve of total production over the course of 5 minutes following the addition of succinate. ROS production values were also normalized to simultaneous rates of O_2 consumption for further analysis.

Cardiac Mitochondrial Function During in vitro Anoxia-Reoxygenation

A different subset of mitochondria were incubated as described above, and glutamate/ malate (10mM/2mM respectively) were added to the chamber followed by 5 mM ADP. Mitochondria were allowed to respire until all oxygen in the chamber had been consumed (~20 minutes). The mitochondria were kept in an anoxic state for 30 minutes, after which 1μM idebenone (n=5) or DMSO (n=5) were added. After 8 min of reoxygenation, to allow the

instrument to match the ambient oxygen concentrations from the beginning of the experiment, the chamber was closed to monitor H_2O_2 and O_2 flux for an additional 5 minutes.

Permeabilized Fiber Mitochondrial Function during in vitro Anoxia-Reoxygenation

Permeabilized cardiac fibers were prepared as described previously [52] with some modifications. The fiber bundles were permeabilized using 50 µg/ml saponin and 0.5 mg/ml collagenase at 4ºC in 1 ml of Buffer A (20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 7.1 mM $MgCl_2$, 50 mM MES, 5 mM ATP, 15 mM phosphocreatine, 2.6 mM CaK_2EGTA, 7.4 mM K_2EGTA, pH 7.0 at 0ºC). After 15 min incubation, the fibers were washed for 15 min in 2 ml of Buffer B (20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 1.6 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4, 2.9 mM CaK_2EGTA, 7 mM K_2EGTA, pH 7.1 at 37ºC) supplemented with 1 µM idebenone or DMSO vehicle control. The respiration measurements with simultaneous H_2O_2 flux detection were performed in MiR05 (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1, and 0.1% BSA) buffer solution using the O2k. To induce anoxia the maximal respiration rate of the sample was stimulated by the addition of substrates, succinate (10 mM) with rotenone (0.5 µM) and ADP (5 mM), and the preparation was left to consume all O_2 in the respiratory chamber (within 10-20 min), thereby entering an anoxic state [53]. After 30 minutes of anoxia, O_2 was reintroduced by opening the chamber. After 8 min of reoxygenation, the chamber was closed and O_2 flux monitored for an additional 2 min. At the end of the experiment antimycin A (2.5 µM) was added to determine mitochondrial-independent residual oxygen consumption (ROX). H_2O_2 flux (ROS flux) was measured simultaneously with respirometry in the O2k-Fluorometer as described above.

Calibrations were performed with H_2O_2 repeatedly added in 0.1 μM steps. 1μM Idebenone (n=5) or vehicle control (n=5) were added at baseline (before addition of permeabilized fibers).

Post-ischemic Cardiac Mitochondrial Function

For this experimental cohort, male Sprague Dawley rat hearts were subjected to ischemia/reperfusion as described above with reperfusion truncated to 30 minutes (to obtain damaged mitochondria before the onset of overt infarction). A separate group of hearts were perfused as normoxic controls. After the protocols, hearts were removed and mitochondria were immediately isolated as described above. The post-ischemic mitochondria were placed in O2k chambers/buffers as described above, and mitochondria respired in State 3 using ADP (1 mM) and glutamate/malate (10mM/2mM), respectively. Following approximately 5 minutes of complex-I-dependent, State 3 respiration, both injured and normoxic mitochondria were treated acutely with 10 μM idebenone (n=8) or a vehicle control (DMSO, n=8). Rates of respiration were monitored for approximately 8 minutes after idebenone treatment.

Cellular Respiration: O2k-Permeabilized Cells

Complementing our cardiac studies, and owing to the difficulty in growing sufficient primary ventricular myocytes in culture, respiration was measured in mouse myoblast C2C12 cells using our O2k Core facility. Cells were cultured to ~80% confluency, counted, resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin, and then centrifuged at 300 x *g* for 3 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in Buffer Z. Buffer Z (2.5 mL)

containing 6 million cells was added to each chamber of the O2k and respiration was measured at 37°C. Cells were permeabilized with 20 μg/ml (final concentration) of saponin added to each chamber. After closing the chambers to maintain a known oxygen concentration a modified Substrate Uncoupler Inhibitor Titration (SUIT) protocol was employed to assess changes in respiration following the addition of various substrates in permeabilized cells. The injection protocol sequence for all experiments was glutamate (10 mM)/malate (2 mM), rotenone (0.5 μM), DMSO (n=5) or 10μM Idebenone (n=4), succinate (10 mM), ADP(5 mM) (all values are final concentrations).

Statistical Analyses

Differences between treatments were assessed using a Student's t-test. In cases where multiple conditions were evaluated a one-way ANOVA was used to determine if a difference existed between conditions. Significance was noted when $P < 0.05$, with values expressed as mean ± standard error of the mean (SEM).

Results

Reduction of Ischemia-Reperfusion Injury with Idebenone

The cardioprotective effects of idebenone are presented in **Fig. 1**. Hearts treated with idebenone at the onset of reperfusion showed significant improvements in cardiac hemodynamics. Left ventricular developed pressure (LVDP) **(Fig.1A)** and maximal rates of contraction (**Fig. 1B**), and relaxation **(Fig. 1C**) were significantly improved with idebenone. Additionally, idebenone-

treated hearts showed a significant decrease in infarct size compared to saline controls ($P < 0.05$) **(Fig.1D)**.

Influence of Idebenone on Mitochondrial RET Production

A number of recent studies [13,54,55] have reported that succinate-derived ROS contribute to ischemia-reperfusion injury through reverse electron transfer (RET). Accordingly, we assessed the impact of idebenone on ROS production evoked by succinate to determine if idebenone acts as a ROS scavenger under these conditions. Mitochondria treated with idebenone showed a significant *increase* in rates of respiration when compared to DMSO control ($P<0.05$) **(Fig. 2 A)**. Similarly, idebenone-treated mitochondria demonstrated a significant increase in the amount of ROS produced when compared to control (DMSO) ($P<0.05$) **(Fig. 2B)**. Succinate-induced RET was consistently higher with idebenone whether the area under the curve of the ROS trace was integrated over 5 minutes **(Fig. 2C)** or normalized to rates of oxygen consumption **(Fig. 2D)**. After the addition of the complex I inhibitor rotenone to decrease ROS production, mitochondria treated with idebenone displayed a significantly higher residual rate of H_2O_2 production **(Fig. 2E)**.

Mitochondrial Function: Insight From in vitro Anoxia-Reoxygenation Models

Determining mitochondrial function after ischemia-reperfusion comes with an inherent limitation that one cannot directly attribute improved mitochondrial function as being a cause or consequence of cardioprotection. Accordingly, we assessed the effects of idebenone in two *in vitro* models to monitor the effects *during* (instead of after) the oxidative insult. In isolated

mitochondria exposed to anoxia-reoxygenation, idebenone treatment improved respiration when given at the onset of reoxygenation, a benefit not observed in permeabilized cardiac fibers **(Fig. 3A and 3B)**. There was no discernible effect of idebenone on raw rates of H_2O_2 production from mitochondria or fibers after reoxygenation, however, rates of H_2O_2 production were significantly elevated after idebenone treatment in normoxic fibers **(Fig. 3C)**. When the rate of respiration was normalized to H_2O_2 production isolated mitochondria showed a modest decrease in total normalized production. In fibers, the A/R-induced increase in ROS production was not significantly attenuated by idebenone treatment **(Fig. 3D)**.

Effects of idebenone on post-ischemic, damaged heart mitochondria.

Studies from post-ischemic mitochondria are presented in **Figure 4**. In these studies, all hearts were exposed to reperfusion (no drug), with the effects of idebeone being determined *after* mitochondrial isolation. Representative traces of oxygen consumption are presented in **Figure 4A**, with quantified data in **Figure 4B.** As expected, I/R induced significant decrements in complex-I-dependent respiration. This decrement in complex-I respiration was significantly improved with acute idebenone treatment ($P<0.05$).

Cellular Respiration

Complimentary studies examining the mechanistic effects of idebenone in cells are presented in **Figure 5**. After permeabilization, rates of respiration in cells displayed a marked increase in respiration, immediately after the addition of 10μM idebenone when compared to

DMSO control **(Fig. 5B)**. Idebenone treated cells also showed a significant increase in the total oxidative phosphorylation (OXPHOS) capacity compared to control **(Fig. 5 A-B)**. Changes in respiration were compared to baseline values to assess differences. Where indicated in **Fig. 5B** idebenone values were compared to a DMSO vehicle control to ascertain the effect of treatment.

Discussion

In the present study, we assessed the cardioprotective effects of idebenone treatment through the lens of mitochondrial function. Myocardial injury after I/R is a multifaceted insult encompassing a host of factors that stress cellular bioenergetics [9,20,56,57]. Complex I is significantly affected by both ischemia and reperfusion. In the last two decades, numerous studies have sought to attenuate mitochondrial dysfunction and mitigate ROS production, with the overarching goal of reducing the ROS burden to restore bioenergetics [2,21]. Among the strategies tested to reduce ROS at or around CI, the use of proposed scavengers/anti-oxidants [26,31,45,58–64], or transient CI inhibition [65–69] to reduce RET have advanced as effective pre-clinical treatments for I/R injury. Despite these advancements, in clinical studies most ROS scavengers do not appear to translate in patients [34–36,57].

In this study, we utilized the benzoquinone idebenone, a compound already in clinical use, for a new indication, cardiac I/R injury. Idebenone was evaluated across injury paradigms, testing the hypothesis that it would significantly improve both functional and bioenergetic capacities in I/R injury. Herein, we provide new insight that idebenone can mitigate mitochondrial damage and bioenergetic dysfunction following I/R. We also shed new light onto idebenone's potential

protective mechanism, demonstrating that it directly stimulates the ETS downstream of complex I but has no discernible ROS-scavenging properties.

Cytoprotection with quinone analogs

Idebenone, originally developed by Takeda Pharmaceuticals for the treatment of Alzheimer's disease, shares structural characteristics with endogenous CoQ10, a vital quinone that facilitates the successive redox reactions between N-/S-linked substrates and complex III. Despite conflicting results on the effectiveness of idebenone treatment in neurodegenerative models [38,70], idebenone and other quinone-like molecules are being investigated in multiple clinical trials for a variety of indications [39,40,71–73]. Compelling evidence suggests that idebenone is more than just a CoQ10 analog, and may have its own unique mechanism of action [42]. Several physiochemical differences highlight the promise of idebenone. Idebenone has a more rapid absorbance than CoQ10 (t_{max} between 1-3 hours [40] vs CoQ10 around 6 hours[74]). The endogenous redox cycler NAD(P)H:quinone oxidoreductases (NQO) displays a preference for short chain quinones [29], and this molecule has a demonstrated ability to directly stimulate complex III in neuronal models [42,43]. Given idebenone's favorable clinical profile and divergence from other quinone analogs, we examined this therapy in the context of cardiac I/R injury.

The reduction in oxidative injury we observed in the reperfused heart with idebenone corroborates other studies using quinone-based moieties in models of diabetic nephropathy [75], ischemia-reperfusion injury [76], and neurodegenerative disease [77]. Previous studies have claimed a ROS-scavenging-dependent mechanism that contributes to the cytoprotection observed

[26,31,76,78]. Contrary to previous insights using quinones, our data indicated that cardioprotection with idebenone does not rely on an antioxidant-based mechanism. By evaluating respiration and ROS production simultaneously, our comprehensive methodology suggest that idebenone is contributing to cardioprotection through a 'bypass' mechanism that circumvents ETS dysfunction and contributes quinone moieties directly to the Q pool. In doing so idebenone appears help maintain the necessary reducing equivalents needed to sustain bioenergetics.

Non-anti-oxidant cardioprotection with idebenone

For more than three decades, idebenone has been lauded as a potent anti-oxidant [79]. To date the rationale and trial design for idebenone has been centered around an anti-oxidant mechanism that has extended from the laboratory into the clinic [33,60,80]. Insightful work from Mordente and colleagues, in cell free systems, found that the efficacy of reduced idebenone as an anti-oxidant varies based on conditions but is comparable to that of other known anti-oxidants [60]. Additional studies in myoglobin and hemoglobin containing solutions demonstrated decrements in lipid peroxidation with idebenone treatment [60] similar to the work of Suno and Nagaoka a decade earlier [79]. In the last two decades, a majority of the work on idebenone's anti-oxidant capacity has been performed in cell-based models or in human clinical trials [38–40,45,60,71,79–83] largely relying on spectrometry approaches or improved clinical outcomes to evaluate efficacy. While the success of idebenone in the clinic, to date, could be attributed to an antioxidant or free radical scavenging ability, limiting it to this singular task fails to incorporate the other redox cycling capabilities this molecule demonstrates in our hands. The lack of free radical scavenging

we observed in our vertically integrated models allows for a more systemic evaluation of idebenone's potential therapeutic efficacy.

From a therapeutic standpoint, the general consensus to protect the post-ischemic heart focuses on reducing the overall amount of ROS [3,21,23,76,84,85]. To our surprise, treatment with idebenone significantly increased succinate-supported respiration in isolated mitochondria and *increased* the total amount of ROS produced. Our observed *increase* in succinate-induced mitochondrial RET, as well as the increase in normoxic cardiac fiber H_2O_2 production after idebenone, compliments earlier work from King and colleagues [86] suggesting that idebenone may interact with a non-physiological site overlapping with the flavin mononucleotide (FMN) site of complex I to produce an unstable semiquinone capable of generating ROS. The production of this unstable semiquinone is further corroborated by our data where rotenone blunted both CI activity and ROS production, yet rates of H_2O_2 production were still significantly higher in idebenone-treated samples. These data suggest that idebenone is not acting as a free radical scavenger in our experimental systems.

Attenuating mitochondrial dysfunction caused by ischemia/reperfusion injury

Complex I damage is known to be one of the early insults to the electron transport system during ischemic injury [9,20,87,88]. Within the first 15 minutes of global ischemia CI activity is diminished and can induce signaling cascades that increase ROS production and damage the mitochondrial phospholipid cardiolipin [20]. In addition to mitochondrial damage, the lack of oxygen during ischemia and the rapid ROS burst at reperfusion significantly damages cardiac function and impacts overall tissue health [89–92]. Current strategies to mitigate ROS damage to

the ETS during ischemia include transient inhibition of CI during reperfusion [65–68], antioxidant approaches [63,76], and cardiac pre- and post-conditioning [93] (see [94] for review).

An attractive strategy to improve post-ischemic energetics is a pharmacological "bypass" mechanism that circumvents dysfunctional CI to maintain bioenergetics and keep ROS emission low [43] [45]. Our data in mitochondria and in cells indicate that idebenone is acting to bypass dysfunctional/blocked CI, and in so doing is improves function and lessens the extent of infarction.

Limitations

The significance of our findings must be considered in light of several limitations. First, determining mitochondrial function in mitochondria isolated after ischemia-reperfusion comes with the inherent limitation that improved function could be a cause and/or consequence of cardioprotection regardless of the mechanism. To address this limitation, we conducted studies in post-ischemic tissue and also exposed healthy mitochondria to an anoxia-reoxygenation insult. Second, measurement of ROS using the Amplex Red system is attractive due to its 1:1 stoichiometry with H_2O_2 but a plethora of other approaches are available for detection of specific radical species that should be explored in subsequent studies. Finally, we do not yet fully understand the therapeutic signaling of a compound that appears to *increase* ROS during the metabolic insults herein. Idebenone treatment may be following a postconditioning-like mechanism involving some aspect of mitochondrial hormesis [93]. Clearly this speculation warrants further investigation.

Conclusion

Despite the prevalence of ostensible "scavengers" to attenuate myocardial injury, we observed mitochondrial protection with idebenone, a clinical-stage compound, demonstrating no evidence of ROS scavenging. Idebenone appears to protect the heart by increasing respiration downstream of impaired complex I. This respiratory stimulation increases mitochondrial ability to combat injury by supplementing reducing equivalents to meet energetic demand(s). These data support the use of idebenone, as a clinically-available compound, for potential testing in the treatment of reperfusion pathologies.

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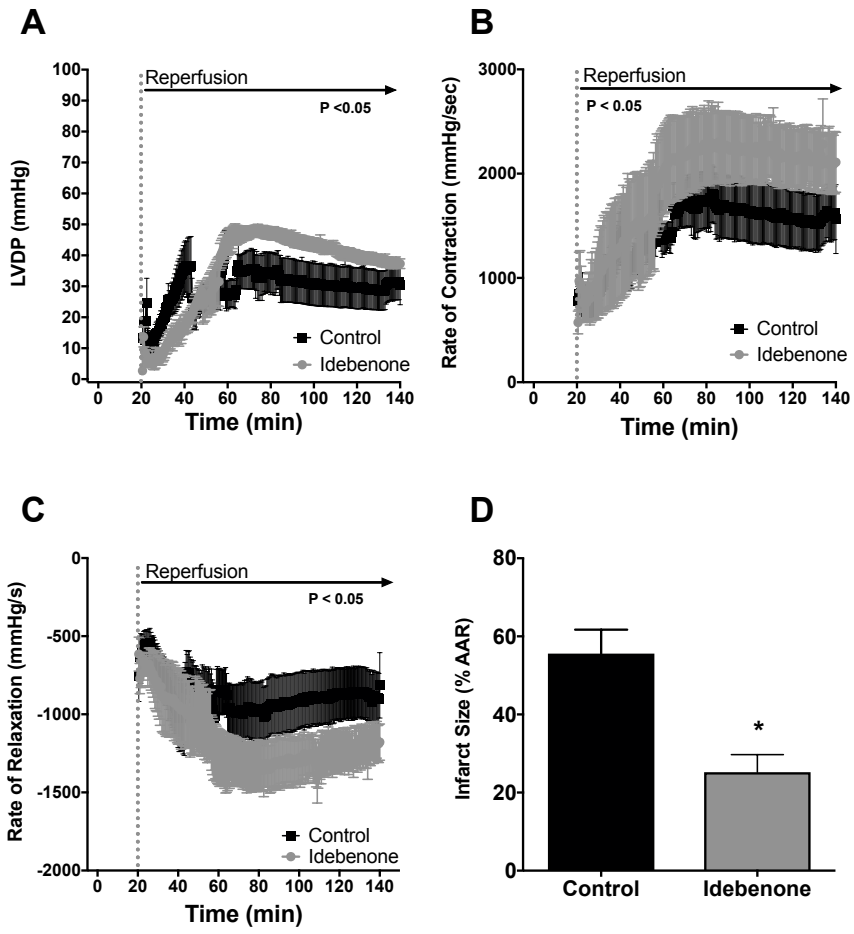

Fig. 1 Cardioprotective effects of idebenone in *ex vivo* perfused hearts. (A) Left ventricular developed pressure (LVDP). Maximal rates of contraction (B) and relaxation (C). (D) Infarct sizes (% area at risk, AAR). *, P <0.05 compared to control. Values are ± SEM.

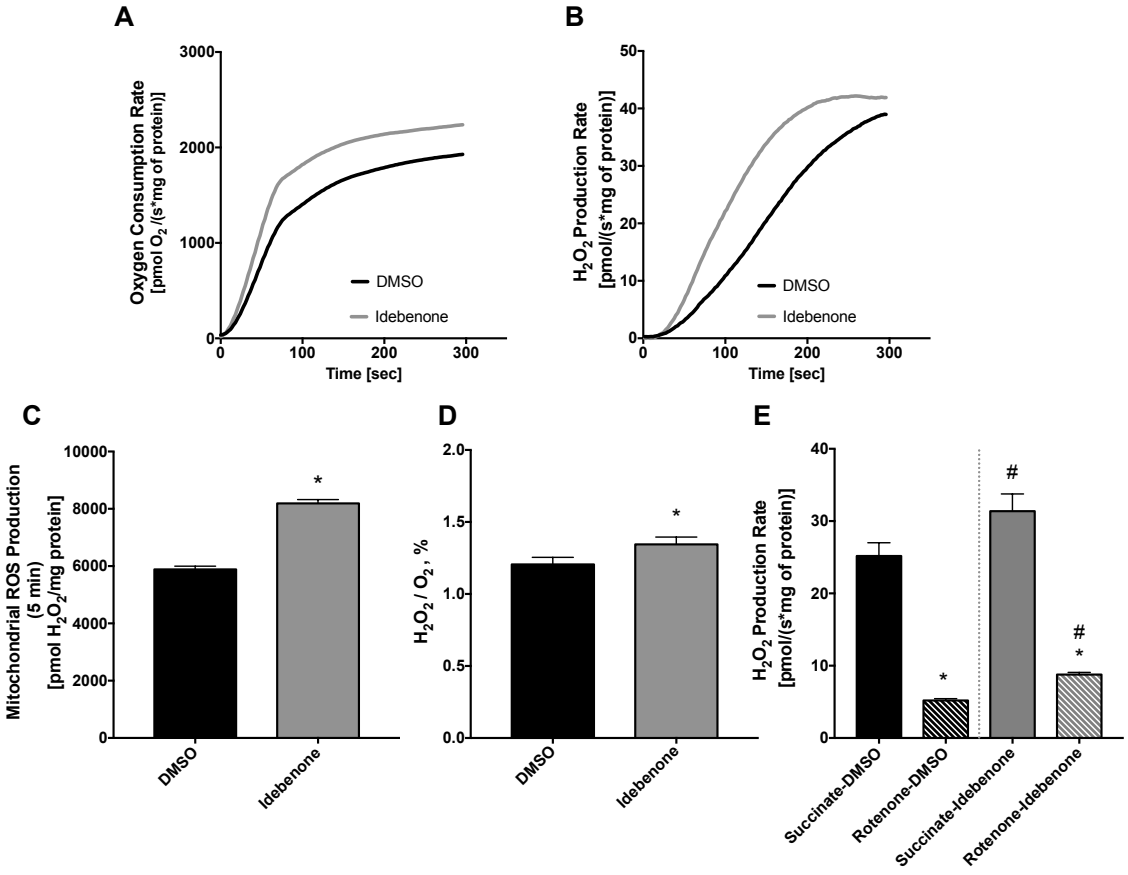

Fig. 2 Mitochondrial RET production was not attenuated with idebenone treatment. (A) Representative trace depicting rates of respiration in isolated mitochondria treated with idebenone or a DMSO vehicle control. (B) Succinate-induced H_2O_2 production in the presence of idebenone. (C) Area Under the Curve for each condition over a 5-minute period. (D) Rates of mitochondrial H_2O_2 production normalized to rates of respiration. (E) Rates of H_2O_2 production after CI inhibition with rotenone. *, different from respective control; #, difference between treatments, $P < 0.05$ compared to control. Values are ± SEM.

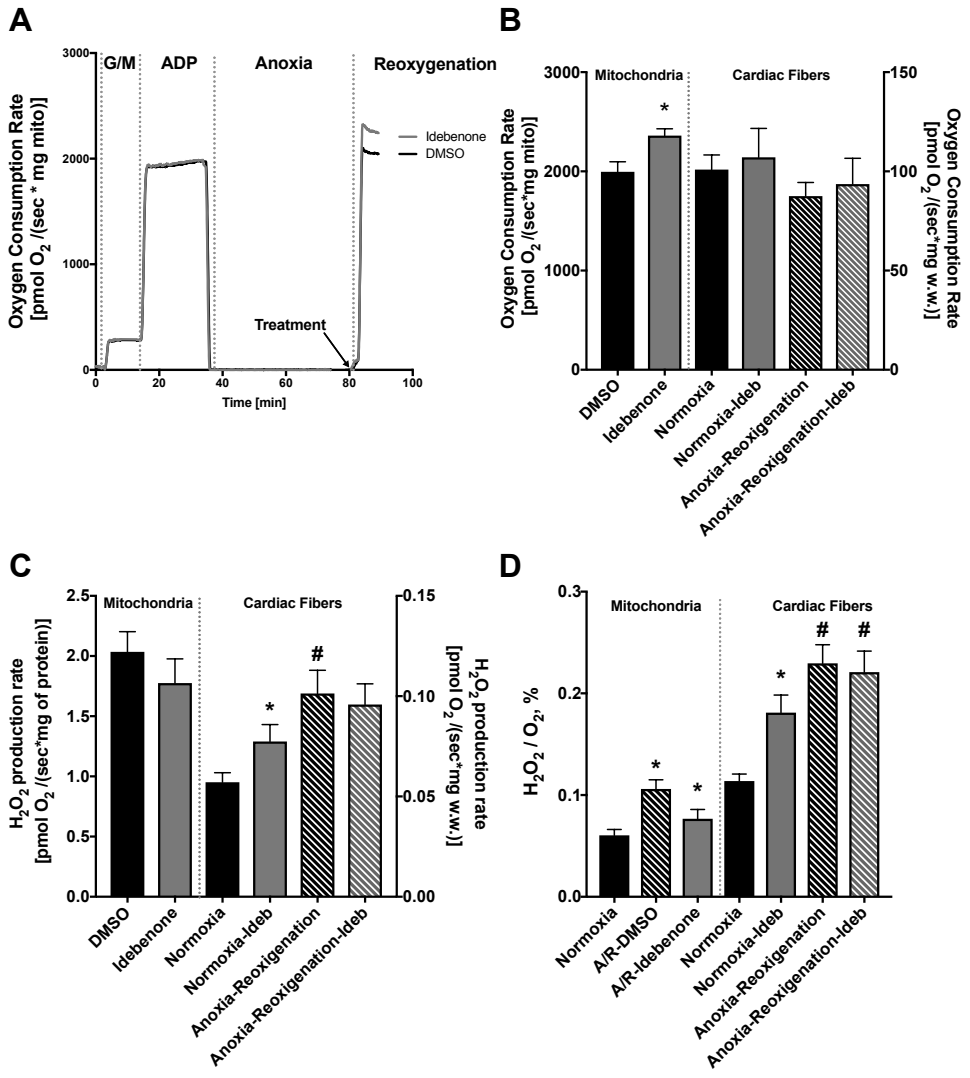

Fig. 3 Effects of idebenone after Anoxia/Reoxygenation injury. (A) Representative trace depicting mitochondrial respiration before, during and after an A/R injury and the concomitant effects of idebenone. (B) Oxygen consumption in isolated mitochondria and permeabilized cardiac fibers after treatment with DMSO or idebenone following the 30-minute A/R injury. (C) Mitochondrial and cardiac fiber H_2O_2 production rates immediately following A/R injury. (D) The percentage of H_2O_2 production after normalization to oxygen consumption rates obtained over the same time course in both isolated mitochondria and cardiac

fibers subjected to an A/R injury. *, P <0.05 compared to control. Values are ± SEM.

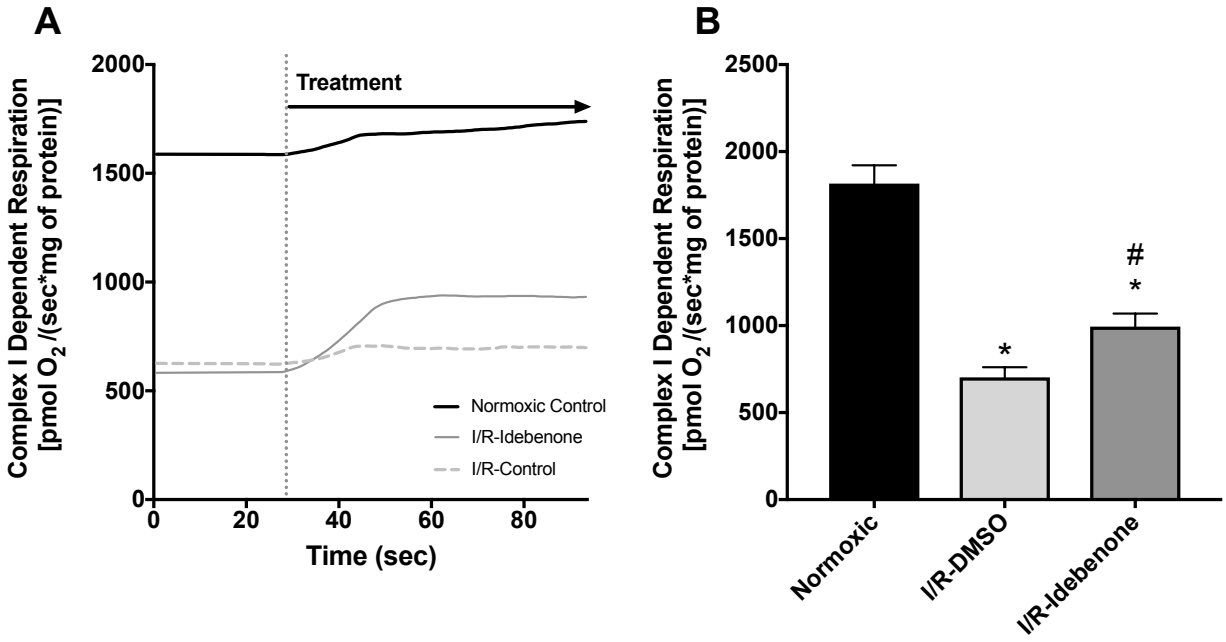

Fig. 4 Idebenone increases complex-I dependent, state 3 respiration in isolated mitochondria after Ischemia-Reperfusion Injury. (A) Representative trace showing State 3 respiration in isolated left ventricular mitochondria from normoxic, I/R injured control, and I/R injured hearts treated with idebenone. (B) Impaired complex I-dependent respiration after I/R hearts was improved with idebenone. I/R-ischemia-reperfusion injured. * different from Normoxic control, # different from vehicle (DMSO) I/R control. P <0.05 compared to control. Values are ± SEM.

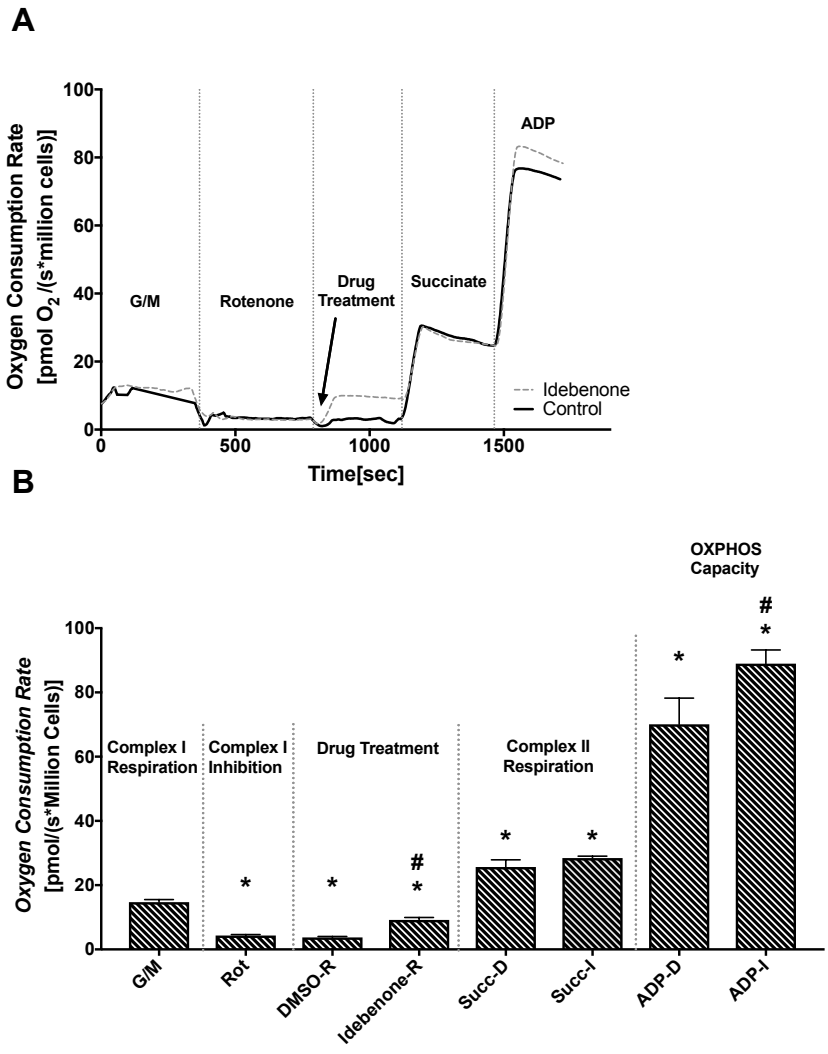

Fig. 5 Idebenone increases oxidative phosphorylation capacity in permeabilized cells. (A) High-resolution respirometry trace of cellular respiration in permeabilized cells depicting a change in respiration after treatment with idebenone or vehicle control. (B) Quantified rates of cellular respiration show increased respiration after idebenone addition as well as in increase in total OXPHOS capacity. G/M-glutamate and malate, Rot-rotenone, Succ-succinate, D-DMSO; I- idebenone, R-reduced with DTT. * different from baseline; # different between DMSO and ideb groups. P <0.05 compared to control. Values are ± SEM.

Chapter 4

Editing subunits of complex I to model mitochondrial dysfunction

Abstract

Mitochondrial dysfunction plays a key role in the progression of a variety of disease states yet no viable treatments currently exist for mitochondrial diseases. This lack of therapeutic treatments is due, in part, to the difficulty in creating experimental models of mitochondrial disease. Targeted disruption of proteins encoded by mitochondrial DNA has been bolstered in recent years by several promising approaches, yet each of these has their limitations. In this study, we utilized Artificial Site-specific RNA Endonucleases (ASREs), a novel mitochondrial RNA engineering platform, and a CRISPR/Cas9 driven approach to create cell-based models of Complex I disease. HEK cells were engineered with CRISPR/Cas9 to knockdown NDUFS2 (HEK293Δndufs2). Slowed growth rate (6.2 ± 0.1 vs 6.9 ± 0.1 cells per flask $\log_{10}$, HEK293Δndufs2 vs parent, respectively) and a decrement in mitochondrial complex-I mediated respiration were observed (13.3 ± 0.3 vs 3.3 ± 0.1 vs 2.9 ± 0.2 pmol/sec*million cells, control vs. D17G8 vs. D2F11, respectively. $P < 0.05$). CRISPR engineered HEK293Δndufs2 cells additionally showed an increased susceptibility to antimycin A challenge and, concomitant with decreased mitochondrial respiration, a significant reduction in total ATP content (168,262 ± 7,725 parent vs. 10,590 ± 859 HEK293Δndufs2, Total Luminescence, A.U.). In a separate cohort of cells, by combining ASRE ablation of a target gene's transcripts with the expression of corresponding human disease allele integrated into the nuclear genome as a transgene, unique cell culture models of human mitochondrial disease were generated. HEK293 cells were transfected with a drug-inducible ASRE designed to decrease the expression of complex I subunit ND1 (ASRE-ND1). In a separate cohort of HEK293 cells, the ASRE-engineered cells were transfected with PiggyBac™

(PB) transposon vectors that carried a cumate-inducible wild type ND1 (PB-ND1) or ND1 gene associated with some forms of Leber's hereditary optic neuropathy (LHON) [PB-ND1(G3640A)]. We next determined the susceptibility of each of the transfected cells to the same antimycin A challenge and saw enhanced toxicity in cells treated with the ASRE (1331 ± 498.3 with cumate vs 778.5 ± 245.4 without cumate, % vehicle control, $P<0.05$). We then employed high-resolution respirometry in permeabilized cells to assess complex I-dependent alterations in cellular bioenergetics. This approach revealed lower complex I dependent respiration after ASRE-ND1 activation (respiration decreased from 13.2±0.7 pmol/sec*million cells to 11.0±0.4 pmol/sec*million cells). Expressing wild type PB-ND1 rescued complex I-dependent respiration to 14.5±0.4 pmol/sec*million cells. Interestingly, expression of the PB-ND1(G3649) LHON mutant in ASRE-ND1 cells did not restore complex I-dependent respiration with these cells respiring at 12.2 ± 0.5 pmol/sec*million cells ($P<0.05$ significantly lower than ND1-rescue cells and no different from ASRE-ND1 cells alone). These data highlight the feasibility of creating cell models of mitochondrial disease using both the ASRE platform and a CRISPR based approach. We anticipate this technology will lead to the development of new screening platforms for emerging therapeutics.

Introduction

Dysfunctional mitochondria and altered bioenergetics are at the heart of a number of disease states[1–8] yet viable, clinically validated, treatments to address this imbalance are virtually nonexistent. The broad range of symptoms that are often masked by other health issues, coupled with the lack of reliable biomarkers makes diagnosing mitochondrial diseases difficult[8–12]. It has been well established that maternal inheritance is the primary source of mutations affecting mitochondrial genes[13,14]. The method by which these mutations are expressed (Mendelian genetics vs population (non-Mendelian) genetics), however, are vastly different[15,16]. The proportion of autosomal recessive or dominant mitochondrial alleles from the nucleus or hetero/homoplasmic expression of mitochondrial DNA (mtDNA), significantly contribute to determining the probability of affected progeny[17] or dictating the severity of the disease, respectively (see [18] for review). This multifactorial contribution to mitochondrial disease progression compounds the difficulty in trying to treat the underlying mitochondrial dysfunction. Despite the rapid advancements in genome editing technologies, the field still struggles to effectively model mitochondrial diseases genotypically. Current approaches to target the nuclear or mitochondrial genome in an effort to knockdown or completely remove the mutated sequence [19–22] have shown efficacy in reducing the mutated mtDNA burden, but have done little to establish a reliable model of mitochondrial disease. Here we provide a new method for modeling mitochondrial disease, not as a replacement for, but as a potential additive to, current genome editing technologies in an effort to better understand the impact of mtDNA mutations and how these mutations impact the therapeutic targeting of mitochondria.

While the vast majority of the electron transport system (ETS) is encoded by nuclear genes, some of the most critical proteins are encoded by the mitochondria [7,23,24] making it difficult to

recapitulate clinically observed mtDNA disease phenotypes in the lab. Through the use of a proprietary technology, artificial site-specific RNA endonuclease (ASRE) [25], to specifically target gene transcripts of mitochondrial DNA as well as CRISPR/Cas9 , to create several stable, inducible, *in vitro* models of mitochondrial CI deficiency. The macromolecular composition of mammalian CI comes from 45 subunits that are encoded by both nuclear[26] and mitochondrial DNA[27–29]. Herein, we target two essential subunits of CI (NDUFS2 and ND1), both involved in the formation of the ubiquinone-binding chamber[28] but each with its own unique characteristics. Through the use of these two cutting edge approaches we demonstrate a significant decrement in mitochondrial respiration, a slowing in growth rate, and an increased sensitivity to mitochondrial toxins for both targeted subunits.

Methods

Cell lines and culture conditions.

Human embryonic kidney cell line 293 (HEK293) were kindly provided by Dr. Joseph Ruiz at Enzerna Biosciences (Raleigh, NC). Media and reagents for growing and maintaining cells were purchased from Life Technologies Corporation (Carlsbad, CA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (by volume) fetal bovine serum and 1% penicillin-streptomycin. Cells were sustained in a humidified incubator at 37ºC and 5% CO_2. A 0.25% trypsin-EDTA solution was used for detachment of cells. One Shot Stbl3 Chemically Competent *Escherichia coli* strain (Life Technologies Corporation) were used to construct the mutagenesis plasmid. Bacteria carrying the plasmids were maintained in Luria Bertani broth or agar for E. coli growth or Ampicillin resistance selection, respectively (LB;

Sigma-Aldrich, St. Louis, MO), and sustained in a humidified incubator at 37ºC and 5% CO_2. The Artificial Site-specific Restriction Endonuclease (ASRE) RNA inhibition system was introduced into the provided HEK293 cells to suppress the *cis*-expression of the native NADH dehydrogenase 1 (ND1) protein. Doxycycline hyclate (Sigma-Aldrich) was used in growth media at 5 μg/ml to induce ASRE activity to suppress the *cis*-expression of native ND1. The resulting strain HEK FRT-ASRE(ND1) was further manipulated by introducing a PiggyBac™ Transposon Vector System (SBI System Biosciences) to *trans*-express the native form of the ND1 or a mutated form of ND1 associated with Leber's hereditary optic neuropathy (LHON) (PB-ND1(G3460A)). Cumate induction solution (SBI System Biosciences) was used in media at 1x concentration to activate the PiggyBac™ system to induce the *trans*-expression of native ND1 or *trans*-expression of LHON ND1. The cumate driven ASRE system and the PB Transposon Vector System were combined to suppress the *cis*-expression of native ND1 and induce the *trans*-expression of either native ND1(HEK FRT-ASRE(ND1); PB-ND1) or the mutated form of ND1(HEK FRT-ASRE(ND1); PB-LHON). (ND1 GeneBank # NC_012920.1:3307-4262, in the same ND1 sequence a G to A nucleotide substitution at position 3460 has been shown to lead to the progression of LHON[30]. A schematic for the workflow of the ASRE system can be seen in Figure 2

CRISPR-Cas9 system

Single guide RNA oligonucleotide design.

The 2061 bp nucleotide sequence of *Homo sapiens ndufs2* (GenBank locus ID BC008868) was used as the base for designing single guide RNA (sgRNA) sequences. Two separate online programs were used for choosing the two mutation sites. The online idtdna program of Integrated DNA Technologies (Skokie, IL) predicted the 1002-bp site of the forward strand as the most reliable for mutating (on-target specificity score 88%). The relevant guide strand predicted by this program for this site was ACGTTTGTTGAACCACATCA located just upstream of the protospacer adjacent motif (PAM) sequence TGG (Figure 1). The online CRISPOR program (http://crispor.tefor.net/) predicted the 970-bp site of the forward strand of *ndufs2* as reliable for mutating with 87% on-target specificity score. The relevant guide strand and PAM sequences predicted by this program for this site were CAGTGGATCCGAGTGCTGTT and TGG, respectively (Figure 1). The top and bottom sgRNA sequences for these two sites (1002-bp site and 970-bp site) were designed and purchased from Integrated DNA Technologies (Figure 1).

Construction of recombinant mutagenesis plasmid.

sgRNA oligonucleotides were resuspended at 100 µM and the top and bottom oligonucleotides were annealed using the following procedure: 1 µl volumes of each oligonucleotide was mixed with 8 µl of nuclease free water. A slow anneal of oligonucleotides was performed on a thermocycler at 95ºC for 5 mins and 95ºC ramping down to 25ºC at 5ºC per min. The annealed sgRNA strands were cloned into the plasmid px458 (Addgene, Watertown, MA). For this purpose, a digestion::ligation reaction was prepared to constitute 2.5 µl Fast Digest

Buffer (Life Technologies Corporation), 16.5 µL nuclease-free water, 1.0 µl empty px458 plasmid at 1 µg/ul concentration (Addgene), 1.0 ul annealed oligos (prepared above), 2.5 µl 10x T4 DNA Ligase buffer (New England Biolabs, Ipswich, MA), 1.0 µl Fast Digest BpiI (Life Technologies), and 0.5 µl T4 DNA Ligase (New England Biolabs). The 25 µl reaction was incubated at 37ºC for an hour followed by 70ºC for 30 min. Competent *Escherichia coli* Stbl3 cells were transformed with digestion::ligation reaction, as described elsewhere[31] and the protocol provided by the vendor. Briefly, 2 µl of digestion::ligation reaction was added into 20 µl of ice-cold chemically competent Stbl3 cells, and the suspension was incubated on ice for 10 min, heat-shocked at 42ºC for 30 sec, and returned to ice for 2 min. Subsequently, 100 µl of LB broth was added to the transformation reaction and the suspension was incubated at 37ºC for 1 h with shaking at 100 rpm. Aliquots of transformation reaction were plated on LB plates supplemented with ampicillin (Sigma-Aldrich) at 100 µg/ml. The cells were plated and incubated overnight at 37ºC. On the following day, individual colonies were picked from plates and streaked on to fresh plates and incubated overnight. Plasmid DNA was extracted from these *E. coli* cells by using a QIAprep spin miniprep kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The presence of the sgRNA segments within the constructed recombinant CRISPR plasmids was validated by DNA sequencing using the U6-Fwd primer (5' GAGGGCCTATTTCCCATGATTCC 3'). The plasmid carrying the sgRNA sequence of the 970-bp site of *ndufs2* was designated as px458-970 while the plasmid with the sgRNA sequence of 1002-bp site was named px458-1002.

Transfection of mammalian cells and isolation of recombinant mutant clones.

HEK293 cells were transfected with the plasmids px458-970 and px458-1002 by using the Xfect Transfection Regent (Takara Bio USA Inc, Mountain View, CA), according to the manufacturer's instructions. Briefly, HEK293 cells grown to 65-75% confluency were harvested and plated onto 6-well plates at 0.5-1.0x10^6 cells in 1 ml medium per well. One µg of plasmid DNA was suspended in 100 µl Xfect Reaction Buffer and 1.5 µl of Xfect polymer was added to the buffer::DNA suspension. The reaction was incubated at room temperature for 10 min and added into the medium carrying HEK293 cells in 6-well plates. After 24 to 48 h incubation, cells were harvested by treatment with trypsin-EDTA, suspended in fresh medium at a ratio of 0.5 cells:100 µl medium (approximately 60 cells in 12 ml medium), and plated in 96-well plates at 100 µl/well. After 5-7 days of incubation, the colonies were inspected for clonal appearance: the wells with rounded colonies radiating from a central point indicated the ones seeded with a single cell. The cells were returned to the incubator and allowed to expand for 2 to 3 additional weeks. A total of 43 wells were identified as potential candidates and were harvested for further work. Portions of harvested cells were saved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in a liquid Nitrogen dewar, a portion was used for extraction of cellular protein, while another portion used for extracting genomic DNA.

5. Preparation of ASRE-HEK293 cells for respirometry and western immuno-blotting.

Frozen cells of FRT-ASRE(ND1); PB-ND1 were washed and propagated in culture medium in 75 cm^2 flasks to 75-80% confluency. Cells were harvested, washed in culture medium, and 1 million cell aliquots were introduced into each of three 75 cm^2 flasks each containing the growth medium supplemented with; (i) no cumate or doxycycline (C^-D^-), (ii) no cumate but with

doxycycline (C^-D^+), or (iii) with cumate and doxycycline (C^+D^+). After two days of growth, cells were passaged into three 150 or 225 cm^2 flasks at 0.35 million cells per flask. After three days of incubation, fresh medium (with or without cumate and/or doxycycline) was added into each flask. The cultures were incubated for an additional four days before harvesting. The harvested cells were washed in growth medium and over 100 million cells from each culture were used for respirometry as described below. Aliquots of 5 million cells from each culture were used to extract proteins for western immune-blotting. Separately, using a similar procedure, the cells transfected with FRT-ASRE(ND1); PB-LHON were expanded, treated with or without cumate and/or doxycycline for seven to ten days, processed, and harvested for respirometry and western immuno-blotting.

Mutant and ND1 expression validation by western immuno-blotting.

Protein was extracted from harvested cells by using the M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific, Rockford, IL), and protein yields were quantified by using the Pierce BCA Protein Assay Kit (Life Technologies Corporation, Grand Island, NY), according to the manufacturer's protocol. Western Immuno-blotting was performed by standard procedures. Briefly, protein preps suspended in Laemmli Sample Buffer (BioRad Laboratories Inc., Hercules, CA) were boiled for 5 min, spun down at 10,000 x *g* for 10 min, and run in 4–20% Mini-PROTEAN protein gels (BioRad). Proteins from gels were transferred to IPFL10100 | Immobilon-FL PVDF Membrane (EMD Millipore, Burlington, MA), and the membranes were blocked with Odyssey® Blocking Buffer (LI-COR Biosciences, Lincoln, NE), incubated at 4ºC overnight with rabbit polyclonal antibodies to NDUFS2 (1:1000 in blocking buffer) (Cat# PA522364; Life Technologies Corporation, Carlsbad, CA), ND1 (1:1000 in blocking buffer) (cat#

19703-1-AP; ProteinTech Group Inc., Rosemont, IL), or a rabbit polyclonal antibody to β-Actin (1:4000) (cat# ab8227, Abcam Inc., Cambridge, MA), washed with phosphate buffered saline (PBS; Life Technologies Corporation), and incubated for 1 h with the secondary antibody IRDye® 800CW Donkey anti-Rabbit IgG (H + L) (Cat# P/N 925-32213; LI-COR Biosciences). The protein bands were detected using an ODYSSEY CLx imaging system (LI-COR Biosciences).

***In vitro* growth rates of strains in culture medium.**

Parent strain HEK293 and the mutant HEK293Δ*ndufs2* grown to 75% confluency in 75 cm^2 flasks were harvested and resuspended in growth media. Aliquots of 50,000 cells were introduced into 25 cm^2 flasks each carrying 5 ml growth media. Cultures were incubated for 4 or 6 days, cells harvested by trypsin-EDTA treatment, and cell numbers were quantified using a standard hemocytometer, in triplicate.

Permeabilized cellular respiration with Oroboros O2k high-resolution respirometry.

Cellular respiration was measured in both permeabilized Human Embryonic Kidney 293 (HEK293) cells and mouse myoblast C2C12 cells using one of five Oroboros Oxygraph 2k ("O2Ks"; Oroboros Instruments, Austria) in the Virginia Tech Metabolism Core Facility. The Oxygen consumption rate (OCR) of strains was determined using protocols described elsewhere [11, 12]. Briefly, the parent type HEK293 and the mutant HEK293Δ*ndufs2* grown for eight days in 225 cm^2 flasks were harvested by treatment with trypsin-EDTA, centrifuged at 300 x *g* for 3 min, the supernatant discarded and the pellet resuspended in Buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM creatine, 1 mM EGTA, 0.05% BSA, and pH 7.1). Buffer Z (2.5 mL). Three million cells were added to each chamber of the O2k and respiration was

measured at 37°C. To achieve permeabilization, 20 µg/ml (final concentration) of saponin was added to each chamber. After closing the chambers to maintain a known oxygen concentration, a modified Substrate Uncoupler Inhibitor Titration (SUIT) protocol was employed to assess changes in respiration following the addition of various substrates in permeabilized cells. The injection protocol sequence for all experiments was glutamate (10 mM)/malate (2 mM), rotenone (0.5 µM), DMSO or Idebenone (10 µM), succinate (10 mM), ADP(5 mM) and FCCP (1 µM) (all values are final concentrations). ASRE treated C2C12 cells follow the exact same protocol for preparation, number of cells per well, and titration injections for respirometry studies.

Miototoxicity of strains and ATP content.

Potential mitochondrial dysfunction of strains was assessed by using the Mitochondrial ToxGlo™ Assay (Promega Corporation, Madison, WI), according to the procedure described by the manufacturer. This assay is based on the measurements of biomarkers associated with changes in cell membrane integrity and cellular ATP levels. Briefly, the parent and mutant cells grown in flasks were harvested by trypsinization and resuspended in fresh medium. Fifty µl aliquots from 200,000 cells/ml suspensions were added to a 96-well plate and incubated overnight. A working stock of antimycin A (AMA) (150 µM) was prepared in a glucose-free DMEM medium (Sigma-Aldrich) supplemented with 10mM galactose (Sigma-Aldrich). Serial dilutions of AMA were prepared in a separate 96-well plate (in galactose containing medium). Following overnight growth, cells were washed with galactose containing medium, and fresh (galactose containing medium) was added into wells at 50 µl/well. 50 µl volumes of the serial dilutions of AMA were transferred from the stock plate to the cells in culture. The wells carrying the vehicle controls received the medium with AMA. Plate was incubated at 37ºC for 90 min. 20 µl volumes of 5X

Cytotoxicity Reagent were added to each well, the plate was incubated at 37ºC for additional 30 min, and the fluorescence was measured at $485nm_{EX}/520\text{-}530nm_{EM}$. The assay plate was equilibrated to room temperature, 100 µl ATP Detection Reagent was added to each well, and luminescence was measured. Values are reported as Total Fluorescence or Total Luminescence for ATP content, in arbitrary units (AU).

Statistical analyses.

The differences between groups were assessed using a Student's t-test. In cases where multiple conditions were evaluated, a one-way ANOVA was used to assess significance. Significance was achieved if $P<0.05$. Values are expressed as mean ± standard error of the mean. Data were plotted and statistical analyses conducted with GraphPad Prism 7 (GraphPad Software, San Diego, CA).

Results

1. Construction of recombinant mutant HEK293Δ*ndufs2 and western-immuno blotting*.

Two separate sites of the gene *ndufs2* on HEK293 genome were targeted for mutating based on the predictions made by two online CRISPR guide tools as the most reliable for mutagenesis with an on-target specificity score of 88%. The recombinant plasmid constructed for mutating the 1002-bp site of the forward strand of the gene was named px458-1002 (Figure 1). A second site for mutagenesis, the 970-bp site of the forward strand of the gene, was also chosen based on the prediction of the other online guide tool (CRISPOR) (Figure 1). The plasmid constructed for mutating this second site was designated px458-970. Transfection of HEK293 cells

with the mutagenesis plasmid px458-1002 produced 22 individual clones each was seen growing from a single cell in wells of the 96 well plates. Out of these 22 clones, six were found missing the approximately 49 kDa protein band. Two of these clones was chosen for further assays and designated HEK293Δ*ndufs2-D2F11* and HEK293Δ*ndufs2-D17G8* (Figure 1). The remaining 16 clones produced protein profiles exactly similar to those of the parent strain suggesting that mutagenesis did not occur. Surprisingly, when the HEK293 cells were transfected with the mutagenesis plasmid px458-970, all 21 individual clones produced protein profiles similar to the parent strain suggesting that none of these clones carried an expected mutation in *ndufs2* gene.

Mutant validation by western-immuno blotting

Six out of 43 total clones, described above, treated with CRISPR/Cas9 against NDUFS2, examined by western immuno-blotting, were found to be missing a protein band of approximately 49 kDa (the native size of NDUFS2) when the respective blots were incubated with the primary antibody to NDUFS2 (Figure 1A). The production of a 42kDa band in the presence of the primary antibody to β-Actin (loading control) confirmed our removal of the 49kDa subunit was not due to an error in loading (Figure 1A). Antibodies for ND1 proteins, extracted from pooled cell populations treated with ASRE, yielded a robust 67kDa band the levels of which were significantly reduced when the ASRE was activated with doxycycline (Figure 3 A&B). The combination of ASRE and PiggyBac™ activation driven by doxycycline and cumate, respectively (Figure 2), resulted in a slight restoration of protein levels for both the healthy ND1 and the LHON (G3460A) mutated form of ND1 (Figure 3 A&B) as evidenced by the ~67kDa band we observe.

***In vitro* growth of strains in culture medium.**

The rate of HEK293 cell growth was dramatically reduced in cells harboring the CRISPR/Cas9 knockdown of NDUFS2. Total number of cells in flasks carrying the parent or the mutant was calculated on the 4th or 6th days of incubation in media. After four days of incubation, the number of cells recovered from the flasks with the mutant was 38% of that from the flasks with the parent strain. After six days of incubation, the number of cells recovered from the flasks carrying the mutant was 18% of that from the flasks carrying the parent strain (Figure 1B). These observations suggest that in culture media, the mutant grow 62 and 82% slower than the parent strain at 4 and 6 days of incubation, respectively.

Mitochondrial respiration.

Having shown that both CRISPR/Cas9 and ASRE treatment alters protein levels enough to effect cellular growth rates we next sought to investigate how much the altered protein levels impacted mitochondrial respiration. In mutant HEK293Δ*ndufs2,* complex I respiration was significantly decreased compared to the parent strain (Figure 1 C&D). Complex II respiration, however, was increased in the mutant, suggesting a possible compensatory regulation in the absence of functional complex I (Figure 1 C-D). In ASRE cells with the ND1 sequence ablated, a clear decrement in CI specific rates of respiration was observed (Figure 3 C-D). Interestingly, the pB overexpression of the same variant from the nuclear transgene shows a slight respiratory improvement while the overexpression of the LHON mutation led to a significant decrease in rates of respiration (Figure 3 D).

Mitotoxicity of strains and ATP availability.

The potential impact on mitochondrial dysfunction of strains treated with either CRISPR/Cas9 or ASRE were assessed by using the Mitochondrial ToxGlo Assay, which is based on the measurements of biomarkers associated with changes in cell membrane integrity and cellular ATP levels. Cell membrane integrity was examined by detecting the presence or absence of a distinct protease activity associated with necrosis using a fluorogenic peptide substrate. The fluorogenic substrate cannot cross the intact membrane of live cells and therefore gives insignificant signal with viable cells relative to non-viable cells. Thus, the cell membrane integrity measured a "dead cell protease activity". In cells harboring ASRE ablated ND1 transcripts mitotoxicity was increased with higher concentrations of AMA (Figure 4 D). Similarly, CRISPR/Cas mutated cells showed an increased susceptibility to AMA insult (Figure 4 C). Total ATP availability of cells was measured by using an ATP Detection Reagent that consists of ATPase inhibitors, a luciferin-containing formulation for ATP detection, and a thermo-stable luciferase. Addition of the ATP detection reagent into cells caused cell lysis and generation of a luminescent signal proportional to the amount of ATP present. In a subset of cells ATP content was assessed in the same wells as the mitotoxicity assay to determine ATP content in the presence of AMA. CRISPR/Cas mutated cells displayed a significant reduction in total ATP content and no effect of AMA on total ATP synthesis (Figure 4 A-B).

Discussion

The need to develop a reliable model of mitochondrial DNA deficits is at an all-time high. Current genome editing techniques have gone a long way in editing individual subunits as well as

removing mutated genes from both nuclear and mitochondrial DNA but have done little to serve as a platform for therapeutically targeting the mitochondria in relation to disease. Here we focus on two subunits of CI, the largest enzyme in the electron transport system and the largest contributor to mitochondrial disease[38]. Through the use of a number of different assays we have assessed multiple parameters that demonstrate a decrement in the function of mitochondria in the presence of three different mutations that recapitulate known clinical phenotypes. The incorporation of these engineered approaches has allowed us to create an established a viable platform for modeling mitochondrial disease.

Engineering dysfunctional mitochondria

The mammalian mitochondrial genome differs from its nuclear counterpart in a number of ways making manipulating its genome even more difficult. The circular mitochondrial genome encodes 37 genes, the majority of which are mitochondrial specific translational machinery (22 tRNAs, 2 ribosomal subunits) leaving only 13 genes that are specific for the five complexes of the electron transport system. In addition to its unique structure mtDNA replication, codon usage, repair and expression all significantly differ from the nucleus[18,39]. Perhaps the most interesting difference between nuclear and mitochondrial genetics stems from the fact that mitochondrial phenotypes follow a more population genetics-based mechanism. With each cell ranging from hundreds to thousands of mitochondria[40] and each mitochondrion containing thousands of copies of mtDNA [18,41], depending on tissue types, there is a relatively high copy number of mtDNA molecules that are simultaneously expressed. This population based clonal expression does allow for a buffer against deleterious mutations, however, cells that do encounter mtDNA mutations do not always yield the same results. A homoplasmic mutation affects all mtDNA

molecules in the same way. A heteroplasmic mutation, by contrast, only affects a portion of the mtDNA molecules[18,42]. The exact degree of heteroplasmy required to express a deleterious phenotype is different for each tissue and largely uncertain, however, some have suggested that 60-80% heteroplasmy is required [18,43]. To further this issue mitochondria are constantly undergoing fusion and fission, making new copies and destroying dysfunctional ones, leading to random segregation of mtDNA molecules [7,18]. This multifactorial difference in expression classification makes modeling and therapeutically treating mtDNA mutations challenging. Several approaches to reduce the levels of mtDNA heteroplasmy through the use of genetic engineering have been undertaken in recent years[19,44–49] with great success.

The era of genetic engineering is still relatively young and continues to change dramatically. While the exact starting point of the genetic engineering revolution may be debated, early work from the 1970's-80's in cloning and simple gene replacement made giant strides in making current genetic engineering a reality[50–56]. Since this early work the field has transitioned from chemical based mutations to RNA interference and double-stranded breaks to more complicated but more reliable engineered protein and nuclease based approaches to edit genomes, the history and multitude of options for these approaches has been reviewed elsewhere[37,57]. Within the context of manipulating components of the mitochondria, both nuclear and mitochondrially encoded, a number of current approaches standout as viable options (reviewed in [58]). Of the contemporary ways to manipulate mtDNA perhaps the most popular approaches include the use of Zinc finger nucleases (ZFNs), and mitochondrial specific transcription activator-like effector nucleases (mitoTALENs). While the CRISPR/Cas9 approach has been hugely successful in the nuclear genome, its success in the mitochondria[54] has been met with strong opposition[44,45]. Arguably some of the strongest evidence against the use of CRISPR/Cas in the mitochondria stems

from the fundamental principles of mitochondrial biology. The fact that mitochondria lack endogenous repair mechanisms coupled with the uncertainty of mitochondrial RNA import present interesting challenges for the CRISPR/Cas system that relies heavily on guide RNA's (gRNAs) to work[44].

Despite the success these technologies have had in editing and eliminating mutant genomes there is still a tremendous need for a reliable model of mitochondrial disease. From a therapeutic stand point being able to remove mutant DNA or reduce levels of heteroplasmy is encouraging but does little to create a model of mitochondrial disease against which pharmacological treatments can be assessed. Cytoplasmic hybrid approaches have had great success in this area[60–63], however, even these approaches have limitations[58]. Given the difficulty in directly modifying the mitochondrial genome to establish a model of mitochondrial disease we sought to facilitate this dysfunction via two separate pathways. Here we embrace these developments and exploit their potential to alter mitochondrial subunits as well as incorporate a new technique to edit mitochondrial transcripts. Our utilization of the CRISPR technology allows for an extreme but insightful knockout of NDUFS2, a well-known contributing subunit to mitochondrial disease. Our novel approach to targeting subunits of the mitochondrial genome using the ASRE system yields, for the first time, the creation of a clinical phenotype *in vivo* by directly targeting mitochondrial transcripts. Contrary to previous methods for editing the mitochondrial genome, the targeting of mtDNA transcripts leaves the mitochondrial genome undisturbed yet provides a more accurate recapitulation of a mitochondrial disease phenotype. We recognize that a confounding variable in the targeting of mitochondrial transcripts is the relatively low rate of protein turnover in cultured cells, however, we aimed to exploit the fact that the accumulation of mutated transcripts helps drive the dysfunctional pathology[64].

Single subunit changes dramatically impact mitochondrial based cellular viability

Oxidative cellular energy production converges on the mitochondrial electron transport system (ETS), with all substrates feeding into the Coenzyme Q pool via complex I (CI) or complex II (succinate dehydrogenase). Among these complexes, CI is by far the largest and is believed to contribute to reactive oxygen species production and subsequent oxidative stress. CI mutations are postulated to be centrally involved in a disease progression and are the largest contributor to mitochondrial specific diseases[38].

Complex I is a multimeric enzyme composed of subunits from both the nuclear and mitochondrial genomes. It is composed of two main portions; a hydrophilic arm and a hydrophobic core. One of the main functions of CI is to reduce quinone moieties at the Q-site to facilitate subsequent redox reactions that culminate in the production of ATP. The site of interaction between the subunits of the hydrophilic and hydrophobic portions of CI forms a pocket or core where quinones are reduced[28]. Of the four subunits that interact to form the boundaries of the Q-binding site ND1 of the hydrophobic arm and NDUFS2 of the hydrophilic arm are perhaps the most important from a structural standpoint. The fact that NDUFS2 and ND1 are located in separate genomes makes them attractive targets for our studies. While we recognize that these are only 2 of the 45 total subunits of the much larger mammalian complex I we feel that their proximity the Q-binding site makes them extremely useful for assessing the impact of mutation on mitochondrial bioenergetics.

Our NDUFS2 CRISPR/Cas9 knockout supports other studies demonstrating the importance of NDUFS2 for CI assembly and respiration[65,66] (REFS for resp). The necessity of NDUFS2

in CI further corroborates the decrement in ATP levels and growth rates we observe in its absence. The ablation of ND1 has recently been shown to have detrimental impacts on CI and subsequent supercomplex assembly[67] highlighting the critical role it plays in coordinating CI function. Our ND1 ablation confirms the importance of the subunit for CI function but furthers our capabilities in understanding the role it has in respiration. While the supposition has yet to be supported through visualization we suspect that alterations to both subunits (NDUFS2 and ND1) change the Q-binding site's physical structure enough to contribute to the decrement in respiration we observe. Both of our NDUFS2 knockouts support the idea of an altered Q-binding site. As expected both NDUFS2 knockout cell lines (D2F11& D17G8) significantly decreased cellular based mitochondrial respiration. Surprisingly, however, in mutant D17G8 we observed an increase in complex II dependent respiration. This increase may suggest a compensatory mechanism within the cells in which CI activity is clearly disrupted. Interestingly, this upregulation in CII dependent respiration rescues total OXPHOS capacity and maximal respiration suggesting a potential endogenous protective response that requires further investigation. The combination of engineering both nuclear encoded subunits as well as mitochondrial transcripts provides a powerful tool to model and evaluate the impacts of mitochondrial disease.

Creation of mitochondrial disease model and potential for therapeutic screening

To date cybrid based models have been the most utilized method for expressing mitochondrial disease[60–63]. Yet as mentioned previously issues with transferred RNA species, proteins and other organelles into recipient ρ0 cells present challenging issues that complicate this approach[58]. In the last few years a dozen or so techniques to modify the mitochondrial genome have been proposed, each with its own set of limitations[58]. Our novel method for modeling

mitochondrial disease in transfected HEK293 cells, a frequently utilized cell type for genomic manipulation studies[63–65], produce a stable, includible model of mitochondrial dysfunction.

Utilizing a CRISPR/Cas9 approach to knockdown NDUFS2 provides a unique opportunity to assess the most significant impact of single subunit changes on mitochondrial disease. NDUFS2 mutations have been implicated in the mitochondrial disease Leigh's Syndrome[69,70]. Leigh's shares a known phenotype with our NDUFS2 knockout in the form of decrements in CI activity[71]. Although the complete removal of NDUFS2 from these cells does not completely model the mutational substitutions in a mitochondrial Leigh's Syndrome it serves as an extreme model of CI deficiency and highlights the limitations of using CRISPR to accurately model mitochondrial diseases. This extreme model provides a viable platform for future therapeutic screening by allowing potential treatment to be evaluated against the "worst case" scenario.

A number of recent studies have investigated the impact of mutated ND1 as it relates to LHON or other mitochondrial diseases[20,62,69–73]. Our novel method using the ASRE technology to knockdown ND1 transcripts to decrease mitochondrial respiration provide a first-in-class approach to modeling mitochondrial disease. Not only are we showing that we can effectively alter mitochondrial genome specific transcripts but that we can precisely model mitochondrial disease phenotypes. This transcript editing approach allows for a more sensitive targeting approach enabling single subunit changes to be made that help facilitate the progression of a disease phenotype. While we can recapitulate the decrement in respiration seen in previous studies the power in our model comes from the added ability to rescue the diseased phenotype with a pB overexpression of the "healthy," non-mutated transcript. This capacity to overexpress any transcript of interest also allows us, for the first time, to express a transcript with a single nucleotide substitution that accurately models a clinical phenotype. The G3460A mutation models a specific

single nucleotide change within the ND1 subunit of CI, one of the three most common LHON phenotypes. While these models have not yet been evaluated with a specific therapeutic intervention we believe that we have created a stable, inducible pair of mutations that can be evaluated separately or in unison to maximize the assessment of potential therapeutic efficacy. Additionally, the potential concomitant expression of a CRISPR targeted nuclear knockout with an ASRE mediated mitochondrial transcript knockdown or overexpression would provide an unrivaled model for studying mitochondrial disease.

Limitations

Despite the enthusiasm for the potentials of this technology we recognize that several limitations must be considered. Early success with this technology has given us hope that it will translate well from an in vitro approach to an in vivo model, however, this translation will undoubtedly require a number of additional hurdles to overcome. *In vivo* utilization of the CRISPR/Cas9 approach is already underway and has yielded some promising results[77], however, more work is required to delivery an *in vivo* model of mitochondrial disfunction driven by the ASRE technology. Secondly, we have focused only on single subunit changes to CI in the absence of consideration for structurally important supernumerary subunits[27,28]. Perhaps the incorporation of altering the supernumerary subunits would shed more light on the structural impacts NDUFS2 and ND1 mutations have on the overall assembly on CI. More detailed analyses of the structural changes after mutation of these subunits is required. Lastly, in order to fully validate these models as potential screening platforms for mitochondrial disease they should be screened against therapeutic agents known to impact mitochondrial function. Compounds like

idebenone, MitoQ, and other coenzyme Q similar, while not clinically approved for mitochondrial disease indications, have shown efficacy in restoring bioenergetics[75–77].

Conclusion

Although many technologies exist to engineer genomes few can directly target the mitochondrial genome and only one exists to both model dysfunction as well as rescue these decrements. By circumventing many of the technical issues found with creating current models of mitochondrial disease our data clearly support the utility of the ASRE technology in recreating clinically relevant models of LHON and ND1 deficiency. Additionally, through the use of CRISPR/Cas9 we have shown the feasibility of removing a single subunit from CI and have modeled mitochondrial dysfunction associated with this loss. Future studies will focus on the combination of these two cutting edge technologies to manipulate both the nuclear and mitochondrial genomes and will enable the ability to create precisely tuned models to address questions the field was previously unable to answer.

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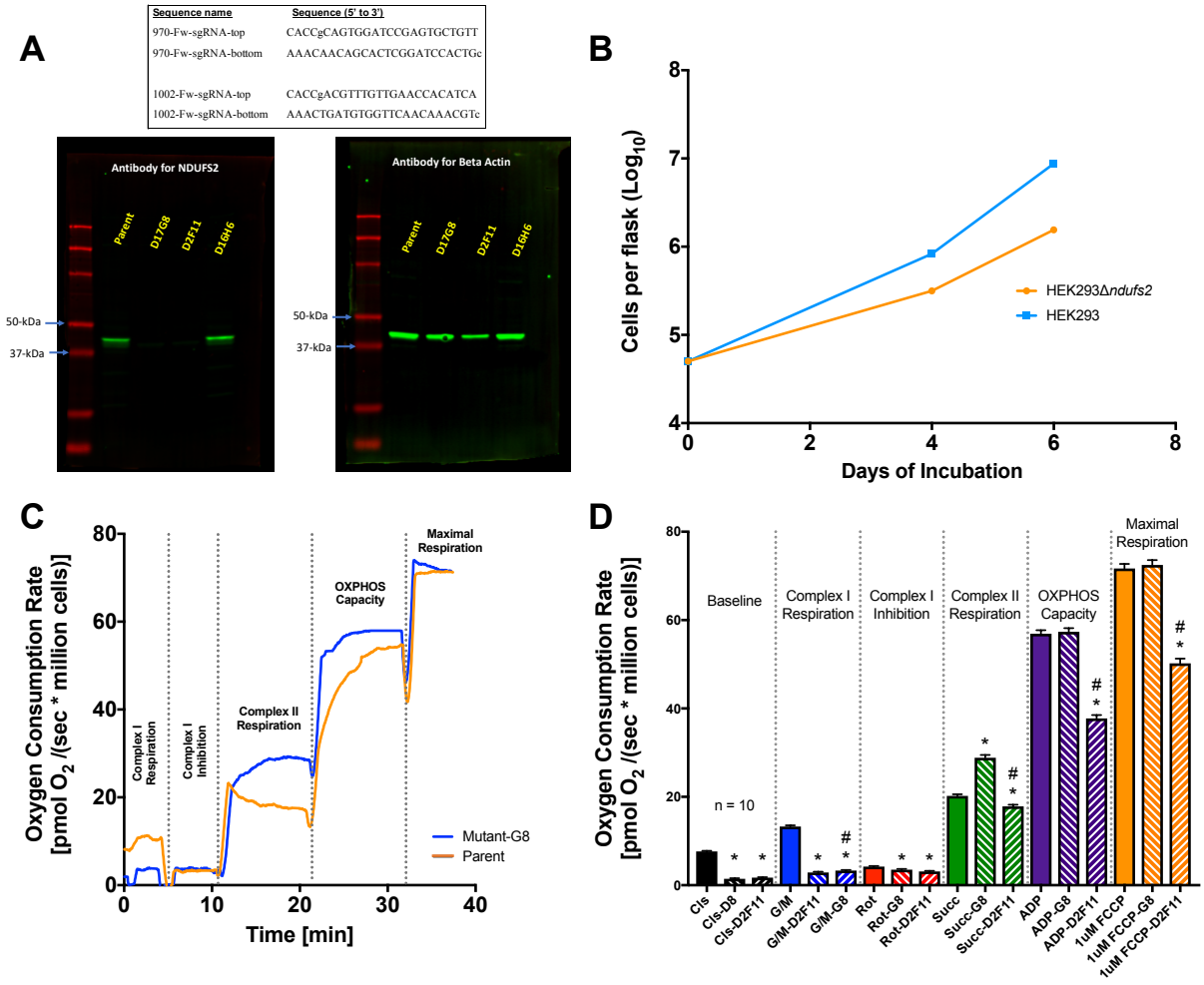

Figure 1. CRISPR mutated NDUFS2 impacts mitochondrial respiration. (A) single guide-RNA oligo nucleotide sequences for mutating the forward strand in two locations within the NDUFS2 gene and corresponding western blots depicting complete knockout of the region of interest in cells transfected with plasmids px458-970 and px458-1002. Beta-actin loading control for each condition. Parent- transfected with plasmid but no sgRNA, D17G8 and D2F11 are separate clones both containing the 1002-bp mutation, D16H6 harbors the 970-bp mutation, and shows no decrement in activity. (B) depicts the decreased rates of growth over 6 days in cells containing the NDUFS2 knockdown. (C) A representative, high-resolution respirometry trace, of HEK293 parent and NDUFS2 mutants. (D) quantified rates of cellular respiration in HEK293

parent and D17G8, D2F11 mutants. * different from control; # difference between mutant groups. P <0.05 compared to control. Values are ± SEM.

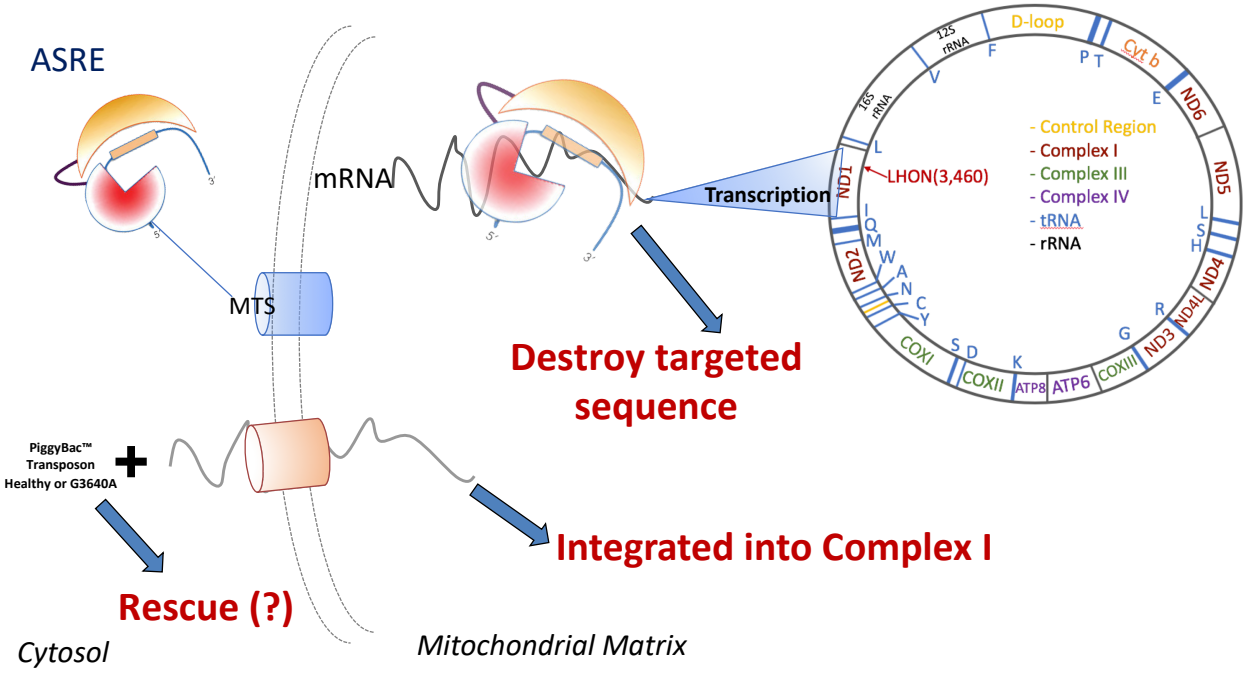

Figure 2. Schematic representation of ASRE technology. ASRE's can be engineered to alter RNA products from any region within the mitochondrial genome. A cumate inducible PiggyBac™ transpon can be utilized to overexpress a sequence of interest.

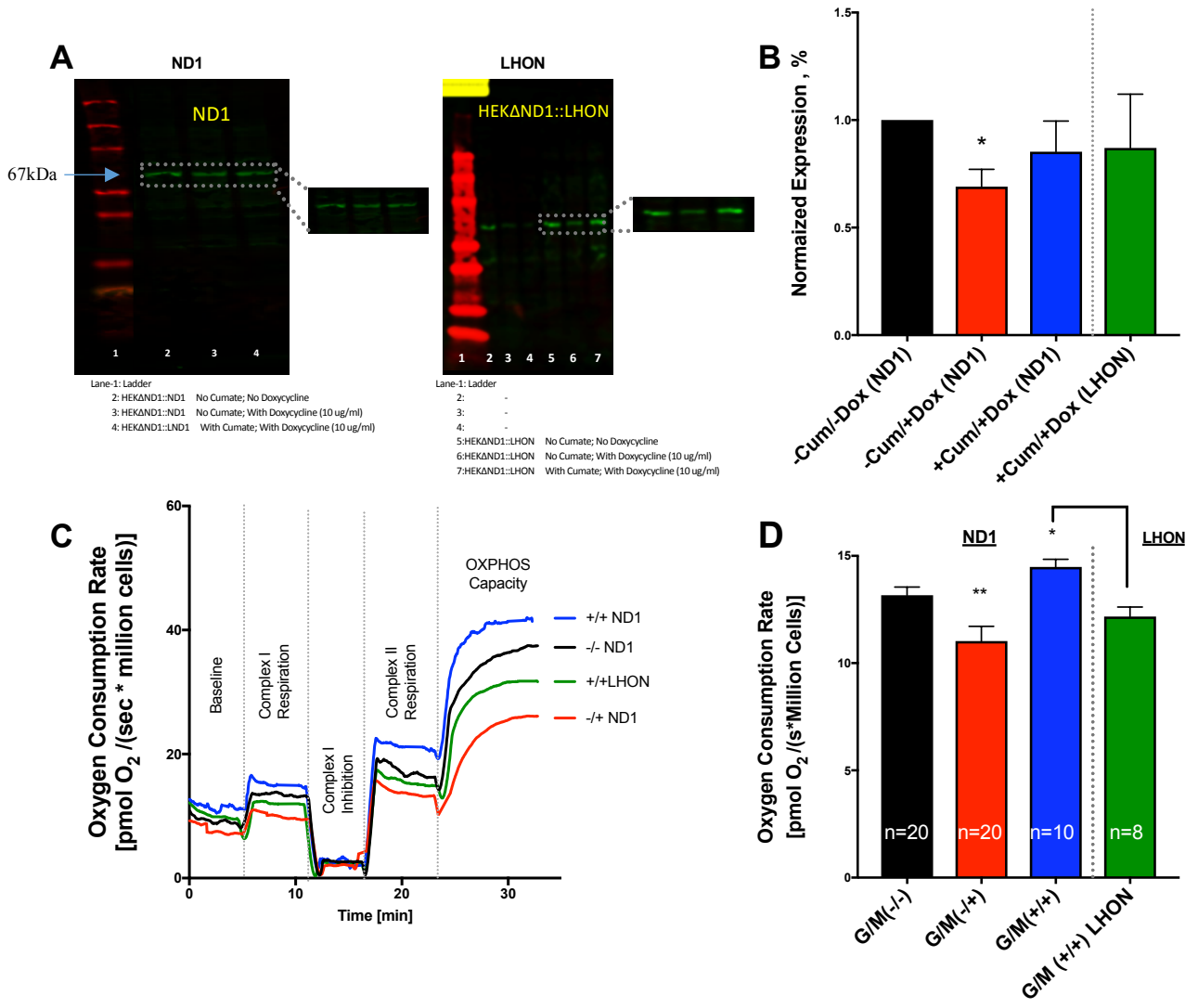

Figure 3. Respiratory Changes in cells driven by ASRE expression. (A) Western blots showing decreased levels of ND1, overexpression of the ND1 variant, and an overexpression of the LHON variant. (B) Protein expression driven by the different inducers in the presence of the ASRE are increased or decreased from the western blots depicted in (A). (C) High-resolution respirometry traces of permeabilized C2C12 cells displaying a difference in rates of respiration after ASRE treatment. (D) Complex I-specific cellular respiration significantly decreased when ND1 RNA is the target of the ASRE technology, a significant increase in rates of cellular respiration when ND1 RNA is overexpressed, and a significant reduction in respiration with the pB-overexpression of

the LHON mutant form of ND1. G/M-glutamate and malate, Cum-Cumate, Dox-Doxycycline, (-/-) no Cumate / no Doxycycline, (-/+) no cumate/ with Doxycycline, (+/+) with Cumate/ with Doxycycline. * are different -/- control; # different between +/+ ND1 and +/+ LHON. * different from control . $P < 0.05$ compared to control. Values are ± SEM.

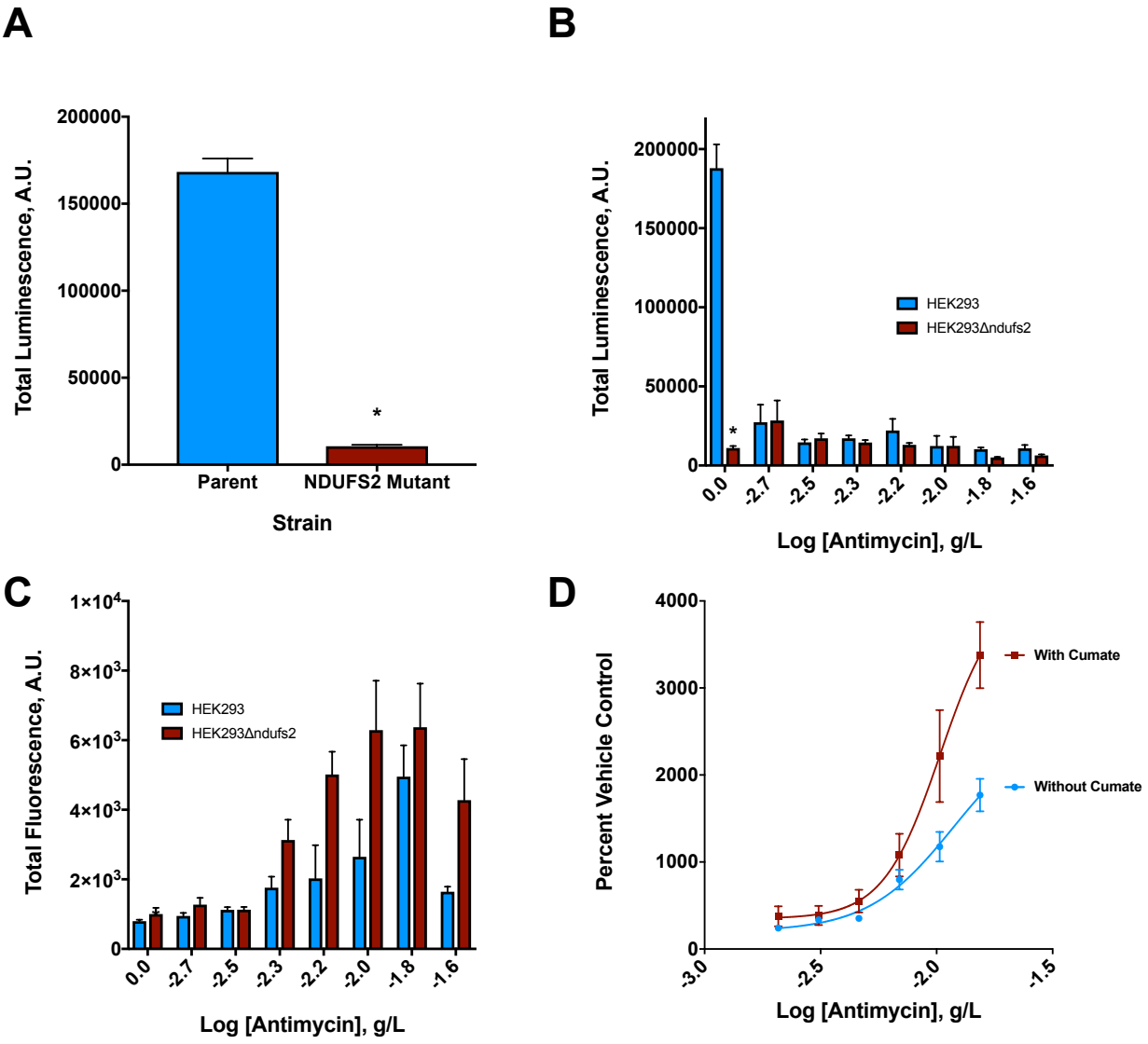

Figure 4. Effect of mutation on ATP production and Sensitivity to insult. (A) Total ATP synthesis is markedly decreased. Effects of AMA on ATP synthesis were unchanged in HEK293Δ*NDUFS2* cells (B) but significantly increased cellular sensitivity to insult (C). For cells treated with an ASRE ablation of ND1 transcripts, AMA sensitivity was increased. $P < 0.05$ compared to control. Values are ± SEM.

Chapter 5

Conclusion

As the impact of mitochondria on health and disease becomes more widely recognized, it is important to understand the precise mechanisms by which these diseases progress. Although we have looked very carefully at specific changes to CI as they relate to ischemic injury and specific subunit changes within CI, there is still much to understand about the implications of CI dysfunction systemically. The steps we have taken towards elucidating some of the inner workings of CI specific diseases have helped to further this knowledge and close the gaps in our current understanding of the role of mitochondria in disease progression. While the work presented here takes meaningful steps toward detailing some of the limitations to currently modeling and understanding CI specific mitochondrial diseases it also provides a number of exciting potential future directions.

In Chapter 2 we discussed the use of a succinate driven RET based model of ROS production as a model to assess novel compound efficacy in I/R based injury. In isolated cardiac mitochondria across a number of different experimental conditions we find that this method for simultaneously collecting ROS production values and oxygen consumption rates to be a reliable physiologically similar model of I/R derived RET induced ROS production. The comparison of conditions helps to validate the use of this model and provides an already validated method ready for others interested in I/R driven ROS production to utilize. The simplicity of this model lends itself to use in any tissue that is challenged by an I/R injury.

As we have shown, I/R injury leads rapidly to CI specific damage in relatively short order. After development of a model to assess CI dysfunction in a chemical based model of I/R, we next

wanted to validate similar results in a physical model of I/R injury. Previous results in both human and animal models of I/R injury have shown potential benefits of using antioxidant based approaches to mitigate ROS induced damage. When we compared one of these proposed antioxidants, idebenone, across a number of different models of CI dysfunction as described in Chapter 3 we found no evidence that this compound was acting as an antioxidant in our models clearly producing ROS. The interesting differences that we observed between the results we obtained in Chapter 2 with idebenone and those in Chapter 3 was that previously idebenone showed some efficacy in reducing total ROS production yet in Chapter 3 idebenone significantly increased ROS production. However, as described in the respective chapters, normalization of ROS production to total oxygen consumption is critical as it completely abrogated this scavenging effect observed in Chapter 2 and maintained its elevated production in Chapter 3 using the same method. We believe that in combination with the appropriate normalization our results obtain in Chapter 3 are more reliable in determining the impact of idebenone on I/R injury. The use of five separate models of CI dysfunction in I/R provided a substantial amount of evidence against the proposed antioxidant based mechanism of idebenone. While there is no doubt that quinone based molecules like idebenone have the ability to scavenge free radicals we believe that our model shows significant proof that under physiological conditions of I/R the cardioprotective mechanism we observe with idebenone treatment comes from a bypass mechanism as opposed to an antioxidant based approach. Briefly, we have shown that in intact hearts, cardiac fibers, isolated mitochondria, and in cells that treatment with idebenone in the presence in CI injury can directly stimulate the ETS to maintain downstream redox reactions and help maintain the bioenergetic demands of the tissue to increase overall cell survivability.

This vertically integrated approach to dealing with CI deficiency mediated by I/R injury provided a great platform for addressing the impact of a known therapeutic on mitochondrial function but we wanted to take an additional step to look outside physical injuries to the mitochondria and evaluate the impact of CI in genetic mitochondrial disease.

CI has been implicated in a number of different mitochondrial diseases ranging from genetic cardiomyopathies to ophthalmic indications. Having demonstrated success in accurately modeling I/R induced CI decrements we next wanted to approach CI dysfunction from a genetic perspective. Chapter 4 demonstrates the utilization of two cutting edge technologies for manipulating subunits of the mitochondria encoded by both the nuclear and mitochondrial genome. Although many technologies exist to engineer genomes few can directly target the mitochondrial genome and only one exists to both model dysfunction as well as rescue these decrements. Here we utilized a CRISPR/Cas9 approach to completely knockdown NDUFS2, a critical component of the hydrophilic arm of CI. We additionally employed a completely novel technology, ASRE, to edit transcripts from the mitochondrial genome. By circumventing many of the technical issues found with creating current models of mitochondrial disease our data clearly support the utility of the ASRE technology in recreating clinically relevant models of LHON and ND1 deficiency. Additionally, through the use of CRISPR/Cas9 we have shown the feasibility of removing a single subunit from CI and have modeled mitochondrial dysfunction associated with this loss. The work in Chapter 4 has demonstrated the exciting possibility of creating precisely tuned models to address questions the field was previously unable to answer.

The use of each of the models described above provide powerful insight into novel approaches to treat CI mediated defects in disease. Along with the establishment and validation of models for further studies we have provided two separate methods for treating CI dysfunction. The

pharmacological approach addressed in studies with idebenone provides a reliable bypass mechanism to defective CI that helps support downstream bioenergetics. Our genetic models provided a rescue of CI transcript knockdown to restore respiration back to health levels via the overexpression of the health transcript. While there is certainly much work left to be done our models of physical and genetic decrements in CI activity have provided a strong foundation for future studies.

The establishment of these foundational models of CI deficiency will benefit from the screening of other mitochondrial-targeted therapeutics. Future studies should focus on the combination of these two cutting edge technologies to manipulate both the nuclear and mitochondrial genomes in cells. The ultimate goal of the use of the genetic approaches is to have a fully functional *in vivo* model of CI disease. Taken together the methods and assays described here will hopefully set the stage for future therapeutic development and the development of additional novel approaches to treat CI mediated diseases.