MITOCHONDRIAL BIOENERGETICS AND CELLULAR STRESS

by

BRIAN P. DRANKA

VICTOR M. DARLEY-USMAR, COMMITTEE CHAIR
ANUPAM AGARWAL
SCOTT BALLINGER
DALE DICKINSON
AIMEE LANDAR

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MITOCHONDRIAL BIOENERGETICS AND CELLULAR STRESS

BRIAN P. DRANKA

MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Mitochondria are responsible for most of the energy produced in human tissues, and this is dependent on the reduction of oxygen (O₂) to water by the mitochondrial respiratory chain. Defects in mitochondrial energy production are now recognized to be involved in diabetes, cancer, cardiovascular disease, and other pathologies. To date, studies of these defects have employed quantification of O₂ consumption in isolated, purified mitochondria. By using this strategy however, the cellular context, role of glycolysis, and normal regulation of mitochondrial function by metabolite availability are lost. Thus, an understanding of how mitochondria function and respond to stimuli in an intact cellular context remains poorly understood. In this thesis, we used a new technology to examine mitochondrial and glycolytic bioenergetic pathways in intact, adherent cells. Interestingly, we found that all cell types consume O₂ under basal conditions and possess a substantial ability to increase O₂ consumption which we have quantified and termed the reserve capacity. This new understanding of O₂ consumption in intact cells led to the hypothesis that the reserve capacity is depleted during cell stress resulting in death. To test this hypothesis, cells were treated with nitric oxide (NO⁻) or 4-hydroxynonenal (HNE), two compounds relevant to the progression of cardiovascular disease. Using endothelial cells, we found that the reserve capacity was decreased in response to both acute and chronic treatment with NO⁻, though the mechanism leading to this decrease differed with time of exposure. Likewise, the reserve capacity was decreased in response to HNE in
isolated cardiomyocytes, and this occurred through yet another mechanism. Additionally, these data indicate the importance of the reserve capacity in response to secondary oxidant stressors. Following non-toxic NO’ treatment in endothelial cells, we demonstrated that secondary oxidative stress exceeded the bioenergetic capacity, and resulted in cell death. Together, these data suggest a novel role for cellular bioenergetics in the control of cell function and indicate that the reserve capacity is an important parameter which modulates the response to oxidant stress. These data also imply that increasing the reserve capacity may be an effective therapeutic strategy extending beyond cardiovascular disease to all cellular bioenergetic derangements.

Keywords: 4-Hydroxynonenal, Extracellular Flux, Nitric Oxide, Oxygen Consumption, Reserve Capacity
DEDICATION

To Anne
ACKNOWLEDGEMENTS

If there is one thing that I have learned during the creation of this dissertation, it is that it is not a feat to be tackled alone. There are many people who deserve my thanks, and I appreciate the opportunity to acknowledge them here. To my mentor, Dr. Victor Darley-Usmar, there are no words of gratitude which accurately describe my appreciation for what you do. The patience you have shown me as you guided my graduate career has been astounding. I am certain that mentoring students is no easy feat, but you make it both a priority and a focus of your research career to mold and shape your students into a form which can rigorously approach the scientific world. Where we have disagreed, you patiently listened to my perspective, even when I did not care to hear yours. Only in hindsight have I learned that is a hallmark of an excellent teacher. I have learned much more than how to do bench science from you. I’ll always appreciate “Friday Beers,” and the philosophical insight they allowed. More than that, I’ll remember the laughs we had recounting old stories. To my committee members, Drs. Anupam Agarwal, Scott Ballinger, Dale Dickinson, Dave Kraus, and Aimee Landar, thank you for serving on my advisory committee for the last four years. I have appreciated all of your direction, and your willingness to challenge me with new ideas and perspectives. I have the utmost respect for all of you, and can only hope to challenge future graduate students in the same way that you all gently guided me.
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<td>°C</td>
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<tr>
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<td>2-deoxy-D-Glucose</td>
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<tr>
<td>ACh</td>
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<td>Aldehyde Dehydrogenase 2</td>
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<tr>
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<td>Amyotrophic Lateral Sclerosis</td>
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<td>CTL</td>
<td>Control</td>
</tr>
<tr>
<td>CuB</td>
<td>B-type Copper</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper/Zinc Superoxide Dismutase</td>
</tr>
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</table>
Cys  Cysteine

Deta NO  \((Z)-1-[2-(2-Aminoethyl)\text{-}N-(2-ammonioethyl)amino]diazen-1-iium-1,2-diolate\)

Deta NONOate  \((Z)-1-[2-(2-Aminoethyl)\text{-}N-(2-ammonioethyl)amino]diazen-1-iium-1,2-diolate\)

DMEM  Dulbecco’s modified Eagle’s medium

DMNQ  2,3-Dimethoxy-1,4-napthoquinone

DNA  Deoxyribonucleic Acid

DPI  Diphenylene iodonium

EcSOD  Extracellular Copper/Zinc Superoxide Dismutase

EtOH  Ethyl alcohol

eNOS  Endothelial Nitric Oxide Synthase

FBS  Fetal Bovine Serum

FCCP  carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone

GFAT  L-Glutamine-D-Fructose-6-Phosphate Amidotransferase

GPx  Glutathione Peroxidase

GSH  Glutathione (reduced)

GSSG  Glutathione (oxidized)

GST  Glutathione S-transferase

h  Hour

H\(_2\)O  Water

H\(_2\)O\(_2\)  Hydrogen Peroxide

His  Histidine

HUVEC  Human Umbilical Vein Endothelial Cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia/Reperfusion</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber’s Hereditary Optic Neuropathy</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial Encephalomyopathy Lactic Acidosis with Stroke-Like Episodes</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<td>ml</td>
<td>Milliliter</td>
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<td>Microliter</td>
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<td>μmol</td>
<td>Micromole</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese Superoxide Dismutase</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mtNOS</td>
<td>Mitochondrial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazoyl blue tetrazolium</td>
</tr>
<tr>
<td>n</td>
<td>Number of replicates</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide Adenine Dinucleotide, Oxidized Form</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide, Reduced Form</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, Reduced Form</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>nmol</td>
<td>Nanomole</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO⁻</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NRVM</td>
<td>Neonatal Rat Ventricular Myocytes</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen Consumption Rate</td>
</tr>
<tr>
<td>OGA</td>
<td>O-GlcNAcase</td>
</tr>
<tr>
<td>O-GlcNAc</td>
<td>O-linked-β-N-Acetylglucosamine</td>
</tr>
<tr>
<td>Oligo</td>
<td>Oligomycin</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome Proliferator-Activated Receptor γ-Coactivator 1α</td>
</tr>
</tbody>
</table>
pH  Power of Hydrogen

$P_i$  Phosphate (inorganic)

PIC  Protease Inhibitor Cocktail

pmol  Picomole

$^{31}$P-NMR  Phosphorus Nuclear Magnetic Resonance

PPAR$\gamma$  Peroxisome Proliferator-Activated Receptor $\gamma$

PUGNAc  $O$-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate

$R^2$  Coefficient of Determination

RNS  Reactive Nitrogen Species

ROS  Reactive Oxygen Species

Rot  Rotenone

RIPA  Radio-Immunoprecipitation Assay

RLS  Reactive Lipid Species

SDS-PAGE  Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

sem  Standard Error of the Mean

Ser  Serine

SIRT1  Silent Information Regulator T1

SOD  Superoxide Dismutase

Thr  Threonine

TIFF  Tag Image File Format

TMPD  $N,N,N',N'$-tetramethyl-$p$-phenylenediamine

TTFA  Thenoyl Trifluoroacetone
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>Uridine Diphosphate-β-N-Acetylglucosamine</td>
</tr>
<tr>
<td>UQCR</td>
<td>Ubiquinone Cytochrome c Oxidoreductase</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-Dependent Anion Channel</td>
</tr>
<tr>
<td>XF</td>
<td>Extracellular Flux</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine Oxidase</td>
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</table>
CHAPTER 1
INTRODUCTION

The role of mitochondria in cellular oxygen consumption was first recognized by Otto Warburg in 1913 [1]. Since then, the mechanisms of oxygen consumption and subsequent energy production have been thoroughly studied, and have earned the mitochondria the nickname of “The Powerhouse of the Cell” [2-5]. Indeed the concept that the mitochondria are the main cellular energy supplier has been accepted for over 50 years now. One mechanism contributing to this concept was proposed by Peter Mitchell whose Chemiosmotic Hypothesis first described the possibility of coupling energetically favorable processes to less favorable ones in a chain that could ultimately result in the production of a usable energy source in cells [6]. At present, much has been elucidated regarding the inner workings of this organelle; however, we are just beginning to understand the importance of energy production in control of cellular fate and function.

The mitochondria are now recognized as dynamic structures capable of fusion and fission to create new mitochondria [7, 8], signaling to the cell during growth [9], adapting to changes in oxygen in the cellular environment [10-14], and controlling cell fate through programmed death [15-17]. The connection between mitochondrial bioenergetics and the control of cell function has been a major focus of my research, and comprises the data that I will present in this thesis. Importantly, the ability to measure mitochondri-
al function in intact cells has recently been expanded. This thesis includes our advances in knowledge regarding mitochondrial function in isolated, intact cells. This is followed by three specific datasets that demonstrate the utility of these new analysis techniques. Furthermore, these data begin to probe the role of mitochondria in several models relevant to cardiovascular disease. Previously, examination of mitochondria in a disease model system required a well-characterized animal model from which these organelles could be isolated. While this approach has led to significant advances in our understanding of how the mitochondria function over the last 40 years, limitations in throughput and sample size preclude the ability to study molecular mechanisms contributing to disease related to the mitochondria. Nevertheless, this approach has demonstrated a role for mitochondrial dysfunction in the pathogenesis of many diseases [18].

MITOCHONDRIA IN DISEASE

The role of mitochondria in disease progression is only now becoming clear. One of the first demonstrations of a disease with an explicit defect in mitochondrial DNA was in the description of Leber’s Hereditary Optic Neuropathy (LHON) [19]. This landmark study established involvement of mitochondrial DNA (mtDNA) mutations in a LHON pedigree which presented with central vision loss and cardiac dysrhythmia—known hallmarks of this disease [20, 21]. Importantly, the LHON phenotype was well characterized prior to demonstrated involvement of the mitochondrial DNA, and known to be entirely maternally inherited, but affecting both men and women. This, along with pathology that involves highly energetic tissues, i.e. the heart and nervous system, led the authors to suspect the mitochondria as a key mediator of this disease [19]. Since this discovery,
many other mitochondrial diseases have been described [22-26]. We know now, however, that the mitochondria are implicated in most diseases, and importantly that mitochondrial involvement does not require mtDNA mutation. There are multiple plausible explanations for this widespread involvement. First, as mentioned above, the mitochondria are major producers of energy in the cell. Deficits in energy production are thus translated to other cellular processes that require energy for proper function. These processes may include ion pumping, which is especially critical for maintaining homeostasis in neurons and other cells, molecular motors such as those that enable muscular function, and the synthesis of molecules for intracellular communication. The loss of any one of these pathways would be largely detrimental to an organism's function. Additionally, the mitochondria are uniquely poised as regulators and integrators of intra- and inter-cellular communication. Seminal discoveries of the necessary and permissive role of the mitochondria in diverse cellular processes have brought into view a role for the mitochondria in responding to viral infection [27], growth signaling [9, 28], and death signals [17, 29-32]. Importantly, the mitochondria are also responsible for retrograde signaling to the nucleus [33]. Involvement in the intrinsic apoptotic death pathway through non-effector proteins such as the Bcl-2 family members Bax and Bak are a typical example [34-36], but also may include regulation of cell-cell junctions [37-40], proteosomal activation [41], kinase activation [42, 43], and oxygen sensing [44].

The central involvement of the mitochondria in the above-mentioned pathways thus uniquely positions this organelle to control cellular function and respond to disease stimuli. Figure 1-1 demonstrates this concept schematically. Listed in this figure are just
Figure 1-1: The mitochondria have a role in the progression of many pathologies. Mitochondrial dysfunction has been described in diseases as diverse as neurodegeneration and cardiovascular disease. This thesis describes the effects of multiple model compounds on functional readouts of mitochondrial bioenergetics. The focus has been specifically on cardiovascular disease, but the results presented here have broad applicability to other diseases as well.
four of the many diseases that the mitochondria have been suggested to have an involvement in. The role of mitochondria, and specifically mitochondrial bioenergetics is now widely accepted in the pathogenesis of cardiovascular disease [45]. Mitochondria have been known to have defects in cancer since Warburg first proposed that cancer cells rely more heavily on glycolysis than normal cells. However, increasing interest is being paid to these organelles as an understanding of mitochondrial bioenergetics gains new ground in this field. Cancer metabolism is thus growing as a potential therapeutic target, and area of intense research interest [46-48]. Similarly, mitochondrial dysfunction is an important feature of Type 2 Diabetes Mellitus. Impaired insulin signaling in the skeletal muscle and other tissues leads to the depression of mitochondrial function and subsequent energy production. Importantly, thiazolidinedione therapy, one of the current front line drugs for the treatment of Type 2 Diabetes, has been reported to protect mitochondrial membrane potential from oxidative stress [49, 50]. As a class, these drugs are known agonists of the Peroxisome Proliferator-Activated Receptor γ (PPARγ). PPARγ agonists have known stimulatory effects on mitochondrial function and biogenesis [51]. Thus one mechanism of action of these drugs may be to improve mitochondrial function in the peripheral tissues of patients with Type 2 Diabetes [52, 53]. Lastly, Neurodegeneration is a broad class of diseases that includes Alzheimer’s Disease, Parkinson’s Disease, and Amyotrophic Lateral Sclerosis (ALS, or Lou Gehrig’s Disease) among many others. These diseases are typified by degeneration of neurons or the myelin sheaths surrounding those neurons. These diseases often result in ataxia, or motor dysfunction, and dementia. Defects in mitochondrial fusion and fission have been reported in neurodegenerative diseases [54, 55], and compounds known to inhibit the mitochondrial electron transport
chain are used to induce Parkinson’s-like disease in mice [56]. Each of these four diseases are broadly known to involve the mitochondria for reasons other than direct defects in, or damage to the mitochondrial DNA. Importantly, these examples are merely windows into the larger view of the mitochondria in a central role for not only the development of disease, but for therapeutic potential as well.

MITOCHONDRIA AND OXYGEN CONSUMPTION

Polarographic Measurements of Oxygen

Until recently, mitochondria have largely been studied through polarographic respirometry. The basis of this technique is shown schematically in Figure 1-2. The nature of mitochondrial oxygen consumption was described in the mid-1950s by Chance and colleagues [57, 58]. Later, this technique was standardized to yield the trace depicted in Figure 1-2. This style was first described by Hackenbrock, which divides mitochondrial respiration into 5 respiratory States [59]. These States are indicated in the Figure 1-2 by small encircled numerals. Polarographic experiments are performed by adding mitochondria to a buffered, stirred solution in a sealed chamber that contains an electrode sensitive to oxygen. As the mitochondria consume the oxygen dissolved in the buffer, the concentration decreases. In the presence of mitochondria alone, there is little if any consumption of oxygen, and this is termed State 1 respiration. Upon addition of mitochondrial substrate, an increase in consumption is measured as the oxygen concentration decreases. In the example shown in Figure 1-2, succinate is listed as the mitochondrial substrate, but these measurements are often performed using a saturating combination of glutamate and malate to drive Complex I or ascorbate and N,N,N',N'-tetramethyl-p-
Figure 1-2: Respiratory states and oxygen consumption in isolated mitochondria. Conventional analysis of mitochondrial function relies on monitoring oxygen consumption over time in response to various pretreatments. In the absence of substrate and ADP, mitochondria will consume very little oxygen, and this is termed State 1. Once a respiratory substrate is added (succinate in the figure above), some O₂ will be consumed, and this is typically ascribed to proton leak occurring across the mitochondrial membrane. This is termed State 2 respiration. Upon the addition of saturating concentrations of ADP, the mitochondria consume O₂ at the maximum possible rate, and this is termed State 3. Once the ADP has been depleted, the mitochondria enter State 4, which is functionally equivalent to State 2. Finally, upon reaching anoxia, the mitochondria enter State 5. The above figure is adapted from the paper by Hackenbrock [59].
phenylenediamine (TMPD) to feed electrons to Complex IV by way of cytochrome c. This increase in respiration is termed State 2. Once State 2 is measured, saturation concentrations of ADP are added to stimulate uninhibited consumption of oxygen at Complex IV, and the concentration of oxygen drops dramatically as the mitochondria now consume it as quickly as they can. This maximal respiration is directly related to the removal of any inhibition of respiration by inadequate ADP. In the presence of high amounts of ADP (as is the case in this measurement) this is termed State 3 respiration, and is one of the major parameters identified using this method. Once the ADP has been depleted, the mitochondria enter State 4 respiration. This respiratory state is analogous to State 2 respiration, and they are often used interchangeably. The ability to use these values interchangeably is due to the similar lack of oxygen consumption related to ATP production at Complex V in these two respiratory states. Finally, upon reaching anoxia, the mitochondria are said to be in State 5 respiration. This pattern of oxygen consumption has been a hallmark of mitochondrial bioenergetic studies for over 50 years now [59]. In combination with enzyme kinetics assays of mitochondrial complexes, the study of defects in specific mitochondrial complexes can be achieved. These techniques have allowed many of the above-mentioned discoveries to be made with regards to the mitochondria involvement in diseases. One important limitation with this technique is in the requirement for a large amount of material on which to perform these measurements. Additionally, the removal of the mitochondria from its native cellular space, and the requirement for saturating substrate conditions precludes the ability to understand mitochondrial oxygen consumption in a cellular setting during disease pathogenesis. These
Whole-Cell Bioenergetic Measurements

Understanding the importance of mitochondrial oxygen consumption in an intact cellular context requires knowledge of complete bioenergetic pathways that may play a role. The predominant metabolic pathways in the cell are glycolysis, and oxidative phosphorylation. The use of a Seahorse Bioscience XF24 Extracellular Flux Analyzer has enabled the study of both of these pathways simultaneously. An in-depth discussion of this instrument is given in Chapter 2 of this thesis. Briefly, the XF24 is capable of measuring extracellular levels of oxygen and protons, which can then be correlated to oxidative phosphorylation and glycolysis respectively. The basis for these assumptions is shown in Figure 1-3. In intact cell culture systems, the predominant source of metabolic “fuel” is glucose. Glucose is broken down by glycolysis to pyruvate, which can then be utilized by the Kreb’s cycle to produce NADH for oxidative phosphorylation. As a result, ATP is produced from ADP and inorganic phosphate, and can then be used to drive processes requiring energy. In normal, aerobic cells, a very small amount of lactate is produced from glycolysis. This lactate is known to be the largest contributor to extracellular acidification, and is thus a surrogate marker for glycolysis in these experiments [60]. Additionally, oxygen is consumed through two main pathways in cell culture. Mitochondrial oxidative phosphorylation is the major contributor, but oxygen can also be used by fatty acid oxidation in the generation of Kreb’s cycle intermediates. Importantly, the amount of fatty acid present in cell culture media is very low, resulting in most oxygen
Figure 1-3: Whole cell bioenergetic pathways can be monitored using measurements at multiple points. Measurements of individual components of mitochondrial respiration and glycolytic flux can be made which allows for interpretation of the effects of a given intervention on whole-cell metabolism. Interrogation of the relative contribution of a given pathway shown above can be made through modulation of the progression from one step to another with inhibitors that are designed to specifically block an enzyme, or protein.
consumption in the cell being performed by the reduction of oxygen to water at Cytochrome c Oxidase. These parameters thus allow for a view of the bioenergetic pathways in the intact cell, and allow for examination of the relative contribution of a given pathway using inhibitors or activators of specific steps. Further discussion of the utility of this technique is offered in Chapter 2.

INTERACTIONS OF NITRIC OXIDE WITH THE MITOCHONDRIA

The mitochondria and many of the proteins that it contains are known targets of nitric oxide (Figure 1-4). This figure shows a representative mitochondrial membrane with Complex I (adapted from [61]) and Complex IV (from the PDB file deposited by Yoshikawa et. al [62]). It has been understood for many years that NO' is able to bind the heme a3 of Cytochrome c Oxidase, which is the same site at which oxygen binds [63, 64]. This seminal discovery has led to the view that the predominant function of NO' in the mitochondria is to inhibit this enzyme, thereby controlling the rate of mitochondrial respiration [65, 66]. Since this discovery, a large amount of research into specific functions of NO' in the mitochondria has yielded some alternative findings. Among these is the finding that NO' metabolites can form S-nitrosothiols on Complex I, inhibiting the function of this enzyme and decreasing mitochondrial respiration rate [67, 68].

Mitochondrial Nitric Oxide Synthase

Perhaps one of the largest debates in mitochondrial biology in the last 10 years has been in the existence of a mitochondrial-localized Nitric Oxide Synthase (mtNOS) [69, 70]. Even though there is no specific independent gene for this enzyme identified,
Nitric oxide has been shown to inhibit mitochondrial function through two distinct mechanisms. Indirectly, NO\(^-\) can form S-nitrosothiols on Complex I from higher order nitrogen oxides. This has been reported to inhibit the Complex’s activity. Additionally, free NO\(^-\) can bind to the copper/heme binuclear center of Complex IV in competition with O\(_2\). This is known to dramatically inhibit mitochondrial oxygen consumption and is one mechanism proposed for NO\(^-\)-mediated control of respiration. Complex I structure adapted from [61], Complex IV structure adapted from [62].
the current consensus holds that one of the other isoforms may exist as a splice variant in the mitochondria. Interestingly, the work of Giulivi and colleagues has demonstrated that this isoform is actually neuronal NOS (nNOS) with an additional phosphorylation and myristylation that localizes it to the mitochondrial inner membrane [71]. The largest argument against the mitochondrial localization of NOS comes from the suspected contamination with cytosolic NOS [72]. Even with rigorous isolation techniques, several researchers have argued against the existence of a *bona fide* mtNOS, and attribute NOS activity in mitochondrial preparations to contaminating eNOS or iNOS.

**Nitric Oxide Directly Inhibits Cytochrome c Oxidase**

The potential existence of mtNOS poses great implications for regulation of mitochondrial function and control of respiration as mentioned above. Placing a source of NO’ in such proximity to Cytochrome c Oxidase would allow for very tight regulation of Cytochrome c Oxidase electron flux, and reduction of oxygen. Independent of the presence of a true mtNOS, NO’ is known to significantly impact on mitochondrial function through interactions with Cytochrome c Oxidase. As mentioned above, NO’ is able to directly bind the heme a₃ in the binuclear center of Cytochrome c Oxidase. This heme is also the binding site for oxygen. Importantly, NO’ binds with a relatively high affinity as compared to that of oxygen. Despite this high affinity, the binding of NO’ to the heme in Cytochrome c Oxidase is known to be reversible and released as the enzyme cycles [73]. Recent reports also indicate that NO’ may bind to the CuB, and thus inhibit oxygen consumption at Cytochrome c Oxidase by a non-competitive mechanism [74]. Through these mechanisms, NO’ has been proposed to regulate mitochondrial respiratory rate in a
manner that controls cellular respiration [75-79]. Additionally, our laboratory recently
demonstrated that the mitochondria may provide for NO’ partitioning into the membrane
[80]. This would allow for localization of NO’ near one important site of action.

THE ROLE OF MITOCHONDRIA IN HNE-PROTEIN ADDUCT FORMATION

Another focus of the work described here relates the effects of exogenous admin-
istration of 4-hydroxynonenal (HNE) on isolated neonatal rat ventricular myocytes
(NRVM). HNE has specific chemical properties which allow for Michael-type addition
reactions by virtue of an electrophilic carbon [81]. This allows reaction with nucleophilic
residues on proteins such as cysteine, histidine, and lysine. Doorn and colleagues report
that the order of reactivity is Cys >> His > Lys, owing to the highly nucleophilic nature
of the cysteine [82]. By adducting proteins, HNE can affect their function. This occurs
through inhibition of the active site of enzymes, and causing protein degradation and
turnover [83, 84].

One putative mechanism of HNE formation is shown in Figure 1-5. In this mod-
el, Reactive Oxygen or Nitrogen Species (ROS/RNS) are produced by intracellular ox-
idases such as Xanthine Oxidase (XO), NADPH Oxidase, and inducible Nitric Oxide Syn-
thase (iNOS). Importantly, ROS and RNS are also produced by the mitochondria, and
also play a role here. HNE is commonly produced as an end product of lipid peroxida-
tion. Typically, this compound is produced from the oxidation of arachidonic acid in cel-
lar membranes [81]. It can then be metabolized by one of several pathways. Aldehyde
dehydrogenase 2 (ALDH2) converts the aldehyde on HNE to a carboxylic acid, forming
Figure 1-5: The role of the mitochondria in HNE-protein adduct formation. ROS and RNS generated from either cellular sources such as xanthine oxidase (XO), inducible nitric oxide synthase (iNOS), and the NADPH oxidases, or from the mitochondrial electron transport chain can increase oxidation of lipids in the mitochondrial membrane. This oxidation can lead to the production of 4-hydroxynonenal (HNE), which can in turn adduct proteins at cysteine, histidine, or lysine residues. Adduction can affect protein function, and typically requires degradation of the protein to be removed.
4-hydroxynonenoic acid. Alternatively, Aldose Reductase converts it to an alcohol which is named dihydroxynonene. Either of these pathways form non-reactive compounds that effectively detoxify HNE. Additionally, HNE can be conjugated to glutathione by Glutathione S-Transferases [85, 86]. This conjugate can then either be extruded into the extracellular space, or further metabolized by Aldose Reductase. Lastly, HNE can form adducts with proteins as described above. This protein modification is known to be present in the pathogenesis of cardiovascular disease [87-89]; however, it is unclear whether these modifications are the cause or consequence of pathology. One focus of this thesis is to describe a novel mechanism whereby HNE-protein adducts may serve to cause cellular damage through adduction to proteins involved in control of cellular bioenergetics. HNE has been suggested to directly adduct proteins involved in energy transfer in the mitochondria such as Cytochrome c [90] and Cytochrome c Oxidase [91, 92]. Furthermore, HNE is known to affect mitochondrial proteins that regulate energy status without direct involvement in electron transfer. The Voltage-Dependent Anion Channel (VDAC) and Adenine Nucleotide Translocase (ANT) are both known to be involved in inhibition of oxygen consumption in response to HNE [93-95]; however, these studies have relied on isolated, purified mitochondria in their bioenergetic analysis. Chapter 4 of this thesis discusses the advances made in understanding the response of intact cells to exogenous HNE. A clearer view of the bioenergetic response to exogenous HNE administration will allow for better understanding of the role of this compound in ischemia/reperfusion injury and heart failure.
The mitochondria are uniquely poised to regulate cellular function due to their critical involvement in multiple cellular processes. This involvement results in an ability to control cell death through cytochrome c release to the cytosol, modulate growth through energy utilization, and direct cell movement through actin dynamics and interaction with new protein synthesis machinery. The classical role for the organelle as the powerhouse of the cell is only now being understood in an intact cellular context. New advances in measurement techniques and analysis are yielding novel views into the control of the processes mentioned above by the cellular bioenergetic status.

Many questions are left unanswered in understanding how cultured cells utilize their energetic capacity. The data presented here demonstrate not only the presence of a spare bioenergetic capacity which is available to the cell in times of need, but also that this reserve capacity can be modulated by various cellular stressors. This thesis examines the bioenergetic response to compounds relevant to cardiovascular disease, e.g. nitric oxide and 4-hydroxynonenal. Based on the previous studies mentioned above, we hypothesize that the reserve capacity is a critical modulator of the cellular response to stress. Importantly, the methods necessary to evaluate this hypothesis by examining the effects of NO’ and HNE on mitochondrial function are presented here in Chapter 2. Next, the cellular response to acute NO’ in endothelial cells is examined in depth in Chapter 3. Following this, the effect of HNE on intact neonatal myocytes is presented in Chapter 4. The impact of chronic administration of NO’ on cell function is discussed in Chapter 5. Lastly, a discussion of the importance of the reserve capacity is included in Chapter 6.
along with a discussion of the future directions possible for examining the role of whole-cell bioenergetics in the cellular response to stressors.
CHAPTER 2
METHODS
INTRODUCTION

In this thesis, extensive use is made of a novel technology for assessing mitochondrial function and glycolytic activity in cultured cells. A significant amount of assay development and experimental design was required to begin generating experimental data, and to validate the cell-based models used herein. This chapter first discusses the principle of the instrumentation used in these studies, followed by a description of the cell line validation that is required before any experimentation is performed. Lastly, the methods developed for data analysis are discussed in depth. The chapter concludes with a description of all other methods used in this thesis.

MEASURING CELLULAR BIOENERGETICS USING THE SEAHORSE BIOSCIENCE XF24 ANALYZER

Introduction

Mitochondrial function measurements in cell culture have to date been largely performed using polarographic electrodes sensitive to oxygen concentration in stirred, buffered solution. As discussed in Chapter 1, these electrodes have become the gold standard for assessing mitochondrial function in isolated samples from animal studies. However, recent advances in fluorometric systems to detect oxygen consumption in cell culture have allowed for sensitive and specific measurements of mitochondrial function
to be made with higher throughput than possible with electrode-based systems. This has enabled the elucidation of many responses to cellular stress stimuli that occur at the level of mitochondrial bioenergetics. The most important difference between these techniques is in the maintenance of a cellular context within which mitochondrial dynamics are examined. This is an important distinction from the work previously done in isolated mitochondria, and will be discussed in detail below. Furthermore, the inclusion of a fluorophore which is sensitive to proton levels allows the determination of the contribution of glycolysis to the overall cellular bioenergetic activity. With these advances, a whole-cell approach to bioenergetics allows a much more comprehensive view of cellular responses to stress.

The acquisition of a Seahorse Bioscience XF24 Extracellular Flux Analyzer in January 2008 has allowed our laboratory to examine mitochondrial function \textit{in vitro} in systems that had not previously been explored due to the laborious nature of performing these experiments with other techniques (Figure 2-1). Examining mitochondrial function in Bovine Aortic Endothelial Cells (BAEC) previously required isolation of multiple large plates of cells, and crude mitochondrial preparation using gentle homogenization and differential centrifugation. With the XF24, mitochondrial function can now be measured in a small fraction of the starting material used for differential centrifugation and with high sensitivity and specificity. Most importantly, this can be done with high throughput which is not possible using other techniques. The XF24 measures oxygen consumption and extracellular acidification in 24 samples at one time. Typically, 4 wells are used as temperature correction/background correction wells, leaving 20 wells for ex-
Figure 2-1: A view of the Seahorse Bioscience XF24 Extracellular Flux Analyzer.
This photograph of the XF24 shows the instrument with the cover removed to demonstrate the component parts of the instrument. Arrows indicate the location of the fiber optic bundles that provide illumination to and emission from the fluorophores used in the cartridge plates, the injection manifold that allows for pneumatic delivery of compounds loaded into the cartridges, and the heat sink that helps to maintain constant temperature. Not shown in this photograph are the computer-based touchscreen controller and the assay cartridge/culture plate kit.
Experimental treatments. Moreover, the automation of the instrument allows for multiple experiments to be completed within one day, since the user can pre-program the protocols into the instrument, load the plates, and continue with other experiments.

Figure 2-1 shows a view of the interior of the Seahorse XF24 instrument. The figure is annotated to show the placement of the fiber optics and waveguides used for fluorescence excitation and emission (discussed below), the injection motor, one of two circulating fans that serve to maintain temperature equilibrium within the system, a heat sink molded as a negative of the tissue culture plate to ensure the temperature remains stable, and the cover that is normally present to house the components shown here.

Assays are performed using “FluxPaks” which contain one disposable assay cartridge, one utility plate that is used for rehydration of the cartridge, and the necessary rehydration solution. The Seahorse XF24 technique relies on two fluorescent sensors spotted on the bottom of disposable assay cartridges. The fluorescence of these probes is quenched by either oxygen or protons. This allows real-time, quantitative measurements of oxygen concentration and extracellular pH in a non-destructive and non-invasive manner. Furthermore, this style of assay allows for further experimentation on the same samples once the XF Assay is complete. The precision of measurement of oxygen and proton concentration by this fluorescence technique is achieved through a mechanism that allows for measurement of these parameters in a very small volume. During a measurement, the housing containing the fluorescence waveguides and injection manifold is lowered onto the assay cartridge. The assay cartridges include a spring-like mechanism
which allows the fluorophore-tipped probes to be raised and lowered to different heights in the culture media. By regulating the height of the sensors, precise control of the sample volume above the cells is allowed. For most experiments presented in this thesis, we have used a plate that allows for a transient 7μl volume to be used. Seahorse Bioscience also offers a plate that uses a 28μl volume, and this is useful for cell lines that consume a large amount of oxygen. The plates are termed V7 and V28 respectively. These plates are discussed in further detail below.

The non-destructive nature of this assay technique sets the XF24 analyzer apart from other newly-developed methods of detection of O2 consumption. Recently, another technique for detecting changes in oxygen in the media surrounding cells was described by Luxcel Biosciences [96, 97]. This method uses a similar principle to the Seahorse Bioscience method; however, it requires the use of a dye reagent that is taken up by the cells and subsequently read in a plate reader. This precludes the ability to measure other parameters following the determination of OCR and ECAR.

One of the most important requirements for successful XF Assays that measure glycolytic function as well as oxygen consumption is the use of unbuffered assay medium. The need for monitoring minute changes in the extracellular pH requires the culture medium be changed to an unbuffered medium for the duration of the experiment. To date we have utilized an unbuffered DMEM for all experiments. This DMEM is identical to that used in routine culture of BAEC and other cells, except that it lacks sodium bicarbonate buffer and FBS. The powder base DMEM is purchased from Mediatech (Manas-
sas, VA) and supplemented with 4mM L-Glutamine, 1mM pyruvate, 100 U/ml penicillin and 100 ng/ml streptomycin, all from Invitrogen (Carlsbad, CA). Importantly, any alterations in this formulation would cause results between experiments to differ widely, and care has been taken in the experiments described in this thesis to keep the formulation consistent.

The non-destructive nature of an XF Assay allows multiple measurements of oxygen and pH in sequence, and can thus be used to monitor kinetic parameters of oxygen consumption and glycolysis in response to an intervention of interest. The ability to perform multiple measurements is achieved through the use of an assay cartridge that can be raised and lowered placed on top of the tissue culture plate. This is shown schematically in Figure 2-2 Panel A. This figure demonstrates both the resting position for the cartridge, as well as the measurement position. Notably, the configuration shown here does not occur during an actual experiment, as all probe heads are moved simultaneously, and cannot be articulated individually. Because the probe heads can be raised and lowered on demand, multiple measurements can be made in sequence, and the system can return to baseline between measurements. The XF24 instrument makes a measurement of the oxygen and proton concentration in each well once every 14 sec. Individual measurements of oxygen concentration rate and extracellular acidification rate are made during 2-5 min intervals. During a measurement, the oxygen concentration decreases to the extent to which the cells consume it, and similarly, the pH is decreased as the cells acidify the media through the production of lactate. Data from a single measurement point are shown in Figure 2-2 Panel B. Data for this figure were taken from an actual experiment performed
Figure 2-2: Schematic view of the oxygen and pH measurement technique. Panel A demonstrates a schematic view of the Seahorse Bioscience Tissue Culture Plate with the two possible positions of the read cartridge simultaneously shown. All probe heads are normally positioned together, however this schematic is annotated to demonstrate the relative position of the probe head during resting periods and during the measurement time. Panel B demonstrates the type of data generated by these measurements. Typically, oxygen concentration decreases over time as the cells consume it, and the pH drops as the cells produce lactate from glycolysis. From these concentration measurements, the rate is calculated and expressed as Oxygen Consumption Rate (OCR) or Extracellular Acidification Rate (ECAR). These rates are the standard format for reporting the data throughout this thesis.
in BAEC, and is indicative of the typical traces seen in these cells. The optimal read time for a measurement must be determined for each cell type. This is discussed further below, and is also shown in Table 2-1.

To minimize the detrimental impact of changes in pH and oxygen concentration that occur during the course of an XF Assay, specialized tissue culture plates are used that have a small surface area for cell culture, but a relatively large volume of media over them. This is shown comparatively in Figure 2-3. This figure shows a standard 96-well plate, a standard 6-well plate, and a Seahorse Bioscience V7 Tissue Culture plate. The plates used for XF Assays have the same surface area of a well in a 96-well plate, however accommodate up to 1 ml in volume. This allows for small changes that occur in the 7 μl read volume to be diluted into the much larger volume above the cells following a measurement.

**Optimization of different cell types for analysis in the XF24 analyzer**

Measurement of oxygen consumption and extracellular acidification in cell culture using the Seahorse technique requires empirical optimization of the cell seeding conditions for each cell type prior to experimental analysis. Multiple parameters must be taken into account, the most important of which are the cell seeding density, the elapsed time from seeding to analysis, the total time in culture or passage number, and the culture media conditions. This is especially important for primary isolated cells, as these cell types are even more sensitive to changes in their environment. To date we have optimized conditions for 11 cell lines. These cell lines are shown in Table 2-1 along with the
Figure 2-3: Size comparison of standard tissue culture plates. For comparison of the seeding area and relative volume of standard tissue culture plates, a 6-well, 96-well, and 24-well Seahorse Plate are shown. The Seahorse Bioscience Plate has the surface area of a 96-well plate, but allows for a relatively large volume over those cells. This large area also allows room for injection of compounds from ports positioned above the well.
optimum mix/wait/measure cycle values. In addition, optimum treatment conditions for each cell line, and at each seeding variation must be determined when using the compounds described below for the mitochondrial function assay.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Full Name</th>
<th>Species</th>
<th>Derivation</th>
<th>Optimal Mix/Wait/Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAEC</td>
<td>Bovine Aortic Endothelial Cell</td>
<td><em>Bos Taurus</em></td>
<td>Aorta</td>
<td>2/2/3</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
<td><em>Homo sapiens</em></td>
<td>Umbilical Vein</td>
<td>2/2/3</td>
</tr>
<tr>
<td>MES13</td>
<td>MES13</td>
<td><em>Mus musculus</em></td>
<td>Mesangial Cell</td>
<td>3/2/2</td>
</tr>
<tr>
<td>NRVM</td>
<td>Primary Neonatal Rat Ventricular Myocyte</td>
<td><em>Rattus norvegicus</em></td>
<td>Neonatal Hearts</td>
<td>3/2/2</td>
</tr>
<tr>
<td>ARVM</td>
<td>Primary Adult Rat Ventricular Myocyte</td>
<td><em>Rattus norvegicus</em></td>
<td>Adult Hearts</td>
<td>1/2/1.5</td>
</tr>
<tr>
<td>Hepatocyte</td>
<td>Primary Rat Hepatocyte</td>
<td><em>Rattus norvegicus</em></td>
<td>Adult Liver</td>
<td>2/2/2</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human Embryonic Kidney Cell</td>
<td><em>Homo sapiens</em></td>
<td>Kidney</td>
<td>2/2/2</td>
</tr>
<tr>
<td>RASMC</td>
<td>Rat Aortic Smooth Muscle cell</td>
<td><em>Rattus norvegicus</em></td>
<td>Aortic Smooth Muscle</td>
<td>2/2/3</td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>Mouse Leukemic Monocyte Macrophage</td>
<td><em>Mus musculus</em></td>
<td>Tumor Macrophage</td>
<td>2/2/2</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
<td><em>Mus musculus</em></td>
<td></td>
<td>2/2/3</td>
</tr>
<tr>
<td>Cardiac</td>
<td>Canine Left and Right Ventricle Fibroblasts</td>
<td><em>Canis familiaris</em></td>
<td>Adult Heart</td>
<td>2/2/3</td>
</tr>
</tbody>
</table>

To demonstrate the optimization required for this technique, a full characterization of Bovine Aortic Endothelial Cells (BAEC) is described in this chapter. As mentioned above, the first piece of information required to begin using this method is in determining the appropriate number of cells to seed per well. The XF24 requires the use of specialized microplates to culture cells for measurements, and these are included in the “FluxPaks” mentioned above. The design of the XF24 microplate is unique in that while there are only 24 wells in the plate, the surface area of the wells is designed to match that of a 96-well plate (Figure 2-3). Thus, this allows for a relatively large volume of media to be present over a small number of cells. The importance of this will be discussed in...
further detail below. In the first series of experiments, BAEC were seeded to a density of 20,000 – 60,000 cells per well in the Seahorse Bioscience V7 tissue culture plates. Oxygen consumption and extracellular acidification were measured three times in multiple wells, and the final data point for each density group was plotted as a function of the original seeding density (Figure 2-4). Importantly, the response in both OCR and ECAR is linear with respect to cell number within this cell density range. Linear regression analysis was performed for both the OCR and ECAR. While the $r^2$ values (0.9857 and 0.8888 for OCR and ECAR, respectively) indicate a strong correlation between the seeding density, and the measured rate, it is of note that these regressions do not pass through the origin. We have found that the relationship between the seeding density, and the measured rate for either OCR or ECAR to only be linear within a certain range. Because of limitations in sensitivity of the XF24, OCR and ECAR cannot be measured effectively below an OCR of 20-300 pmol/min and an ECAR of 2.0 mpH/min. These limitations do not typically affect our experiments due to the fact that our experiments are optimized to occur well above these rates. For the remaining experiments in BAEC, 40,000 cells/well was chosen as the optimal condition. This density of cells provided a confluent monolayer within 24 hours post-seeding, and yielded a basal OCR (~120 pmol/min) that allows for both inhibitory and excitatory processes to be examined while maintaining a high signal to noise ratio.

**Development of an assay for mitochondrial function**

One of the largest advances in our understanding of how the mitochondria function in intact cells *in vitro* has come from the development of a functional assay that
Figure 2-4: Measurement of basal oxygen consumption and extracellular acidification in BAEC using the XF24 analyzer. Seahorse Bioscience V7 Tissue Culture Plates were seeded with BAEC (20,000–60,000 cells/well) and allowed to grow for 24 h before the measurement of OCR and ECAR. Linear regression analysis of the data was performed and is shown here. $r^2$ values for the least squares linear regression are 0.9857 and 0.8888 for OCR and ECAR, respectively. Data shown are the means ± sem, n≥3 per group.
measures mitochondrial oxygen consumption. Using well-characterized inhibitors of mitochondrial function we were able to determine the relative contribution of several different parameters of mitochondrial oxygen consumption to total organellar function. This assay was designed to measure six parameters of mitochondrial function: basal oxygen consumption, ATP-linked oxygen consumption, proton leak, maximal oxygen consumption possible at Complex IV, reserve capacity, and non-mitochondrial oxygen consumption. These parameters are distinguished using three sequential injections of mitochondrial inhibitors at the end of an XF assay. First, oligomycin is injected to inhibit the ATP synthase, or Complex V. Inhibition of this enzyme causes a decrease in the measured OCR due to inhibition of ATP synthesis. The difference between the basal OCR and this rate thus yields the amount of oxygen consumption that is ATP-linked [98]. Next, FCCP is injected to uncouple the mitochondrial inner membrane. FCCP is a proton ionophore, and allows for uninhibited movement of protons across the mitochondrial inner membrane. The effect of this is a complete depletion of the mitochondrial membrane potential ($\Delta \Psi_m$), and uninhibited electron flow through the mitochondrial respiratory chain. This leads to a dramatic increase in oxygen consumption, and allows a determination of the maximal oxygen consumption that is possible at Cytochrome $c$ Oxidase (Complex IV). Thus, the addition of FCCP allows for a good estimate of the true value for maximum OCR without dependence on ATP/ADP transport [99]. Additionally, this method for determining the maximum OCR has been in use for many years [98]. The difference between the FCCP stimulated rate and the basal OCR yields an estimate of the reserve capacity of the cells. Reserve capacity is defined as the amount of oxygen consumption that is available to cells to use in times of increased ATP demand, or under stress. Lastly,
antimycin A is injected to inhibit electron flux through complex III. This has the effect of preventing any oxygen from being consumed at complex IV, and thus yields the rate of oxygen consumption that is being consumed at sites other than the mitochondrion [98]. The concentrations necessary to achieve the optimal result for each of these measurements must be empirically determined for each cell line before being used for experiments. This optimization has been performed for many of the cell types listed in Table 2-1, and the optimized conditions are listed in Table 2-2.

### Table 2-2: Optimized Seeding Densities and Concentrations of Oligomycin, FCCP, and Antimycin A.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Optimal Seeding Density (cells/well)</th>
<th>Oligomycin (μg/ml)</th>
<th>FCCP (μM)</th>
<th>Antimycin A (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAEC</td>
<td>40,000&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>HUVEC</td>
<td>30,000</td>
<td>1</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>MES13</td>
<td>10,000</td>
<td>0.3</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>NRVM</td>
<td>75,000</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>ARVM</td>
<td>15,000&lt;sup&gt;2&lt;/sup&gt;</td>
<td>N/A&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatocyte</td>
<td>10,000</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>HEK 293</td>
<td>25,000</td>
<td>0.3</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>RASMC</td>
<td>40,000</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>80,000</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>MEF</td>
<td>40,000</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cardiac Fibroblasts</td>
<td>50,000&lt;sup&gt;5&lt;/sup&gt;</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
<td>N/D&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Experiments in Chapter 5 were seeded at 20,000 cells/well to allow for an extended treatment time.

<sup>2</sup>Seeded into Seahorse Bioscience V28 Plates.

<sup>3</sup>These cells do not respond to oligomycin treatment, and may require digitonin permeabilization in order to achieve inhibition of ATP synthesis by this compound.

<sup>4</sup>The optimal dose has not been determined for these cells.

<sup>5</sup>Experiments were performed the same day with this seeding density.

A representative trace of the data yielded from this type of experiment is shown in Figure 2-5. This figure represents an actual trace from an experiment as described above, but is annotated to indicate the relative contribution of each of the mitochondrial respiratory parameters to the overall cellular bioenergetic function. The example shown in Figure 2-5 is taken from BAEC, and is indicative of the trace seen for 40,000 cells/well. It is
Figure 2-5: Defining mitochondrial function in vitro. A representative time course for measurement of OCR for 40,000 BAEC under the basal condition followed by the sequential addition of oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) with a measurement of OCR and ECAR as indicated. This progress curve is annotated to show the relative contribution of non-respiratory chain oxygen consumption, ATP-linked oxygen consumption, the maximal OCR after the addition of FCCP, and the reserve capacity of the cells. All data are the means ± sem, n≥3 per group.
**Figure 2-6: Measurement of Mitochondrial Function in BAEC using the XF24 analyzer.** The contribution of each of the parameters shown in Figure 2-2 to the total cellular oxygen consumption is plotted. All data are the means ± sem, n≥3 per group. *, p<0.05 vs. Basal; #, p<0.05 vs. Maximal OCR.
apparent from this data that the majority of the basal oxygen consumption in these cells is attributable to the production of ATP. There is a small amount of proton leak in these cells that comprises approximately 10% of the basal oxygen consumption on average. Interestingly, BAEC also possess a substantial reserve capacity that can be measured upon treatment with FCCP. This reserve capacity is seen as a large increase in the OCR when cells are treated with 1μM FCCP. Finally, in each experiment we have noted that there is some amount of oxygen consumption that cannot be prevented upon treatment with antimycin A. Thus the antimycin A-insensitive rate is ascribed to non-mitochondrial oxygen consumption as described above. In BAEC this rate comprises approximately 10% of the total OCR at baseline (Figure 2-5). The data presented in this figure can be separated into the component parts for a clearer graphical representation of the impact of other experimental treatments on these individual parameters. This is shown in Figure 2-6. It is important to note here that the Proton Leak OCR, ATP-Linked OCR, and Non-Mitochondrial OCR should sum to the Basal Rate. Similarly adding these rates to the Reserve Capacity should yield the Maximal OCR as shown in this graph.

**Measuring extracellular acidification to examine glycolysis**

Because the XF24 can measure both oxygen and proton concentration simultaneously, we can also generate a trace of ECAR that matches the experiment described for OCR in Figure 2-6. Experimentally, the ECAR is largely attributed to the flux of substrates through the glycolytic pathway. Incubation of cells with 2-deoxy-D-glucose, a glucose analog that is unable to be metabolized to pyruvate, and a known inhibitor of glycolysis, has been shown to largely inhibit ECAR in several cell types [60]. The trace
Figure 2-7: ECAR is stimulated by modulators of mitochondrial function. BAEC were seeded to 40,000 cells/well and allowed to grow for 24 hours in complete media. Mitochondrial function was examined as described in Figure 2-2, and extracellular acidification was monitored in parallel. A representative time course for measurement of ECAR under the basal condition followed by the sequential addition of oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) with a measurement as indicated. All data are the means ± sem, n≥3 per group.
of ECAR during the mitochondrial function assay is shown in Figure 2-7. Initially, three basal measurements of ECAR are made. This is followed by the sequential injection of Oligomycin (1 μg/ml final concentration), FCCP (1 μM final concentration), and antimycin A (10 μM final concentration) as in the mitochondrial function assay. As shown in Figure 2-7, oligomycin increases ECAR nearly 3-fold in these cells. This is likely due to the inhibition of mitochondrial ATP synthesis that occurs when the cells are treated with this compound. The stimulation of glycolysis is thus a compensatory reaction to the lack of ATP supply coming from the mitochondria. We have noted that FCCP also increases ECAR (Figure 2-7) however the mechanism by which this occurs is unclear. Because FCCP is a proton ionophore, the increase in ECAR seen after treatment with FCCP may be simply related to the pH gradient that is present between the cells and the assay media. Antimycin A has little effect on the ECAR of these cells, though we have noted that it can decrease ECAR in other cell lines. Because of the temporal nature of this experiment, it is also unclear what information is yielded from the ECAR rate post-antimycin A treatment given that it is measured in the presence of FCCP.

**Bioenergetic “Imaging”**

The ability to examine both of the major energy producing pathways simultaneously in cell culture has enabled a new concept in defining bioenergetic status of intact cells. As shown in Figure 2-8, a plot of the OCR and ECAR in response to various stimuli or inhibitors can elucidate the effects on a larger view of the cell’s energetic status. In this experiment, BAEC were treated with increasing concentrations of antimycin A, oligomycin, the complex I inhibitor rotenone, the complex II inhibitor TTFA, or the
Figure 2-8: Bioenergetic profile of BAEC treated with inhibitors of mitochondrial oxygen consumption. BAEC were seeded at 40,000 cells/well and treated with 10 μM Antimycin A, 1 μg/ml oligomycin, 1 μM rotenone, 10 μM TTFA, or 1 mM cyanide, and OCR and ECAR were measured. The resulting effects on OCR and ECAR are plotted as a percentage of the baseline measurement for each treatment. Data shown are the means ± sem. n≥3 per treatment group.
complex IV inhibitor cyanide. The dose that demonstrated a maximal effect is then dis-
played as a percentage of the basal rate. Plotting both OCR and ECAR allows the visual-
ization of the inhibition of oxygen consumption and concomitant increase in glycolysis
that is seen with antimycin A, oligomycin, rotenone, and cyanide (Figure 2-8). Interest-
ingly, TTFA has little effect on either the OCR or ECAR. If the total potential electron
supply to the mitochondrial respiratory chain is considered as coming from either Com-
plex I or II, then this data suggests that there is little contribution to the normal basal
OCR from Complex II. Additional support for this concept comes from the data with ro-
tenone. Figure 2-8 shows that rotenone inhibits OCR to a level near the antimycin A rate.
This suggests that the contribution of Complex I to the basal OCR in these cells is likely
to be at least 90%.

**Not all Cells are Created Equal**

As mentioned earlier in this chapter, we have characterized 11 cell lines to date,
and have found that each cell line has its own distinct pattern in response to this mito-
chondrial function assay. Figure 2-9 shows two additional cell lines that we have charac-
terized in depth. While a complete analysis of the mitochondrial function of these cell
lines is beyond the scope of this chapter, it is important to note that there are large differ-
ences in the degree and relative contribution to OCR of the different oxygen consuming
pathways examined with this technique. For example, primary neonatal rat ventricular
myocytes (NRVM) have a much higher basal rate of oxygen consumption, and an overall
larger respiratory capacity than do BAEC (Figure 2-9). Notably, NRVM also use a
greater proportion of the basal OCR to compensate for proton leak, indicating that these
Figure 2-9: Mitochondrial function differs between cell type. Shown are the mitochondrial function assay traces for three cell lines discussed in this thesis (Panel A). Panel B shows the relative contribution of the Reserve Capacity, ATP-Linked OCR, Proton Leak, and Non-Mitochondrial OCR to the maximal OCR for a given cell line.
cells are somewhat less efficient than the endothelial cells. Rat aortic smooth muscle cells (RASMC) have a metabolic capacity that is more similar to BAEC; however, they display a much lower reserve capacity (Figure 2-9). These parameters indicate that each cell line is different, and that the mitochondrial function must be empirically determined for each new cell line used. The three cell lines described in Figure 2-9 are representative of the disparate cell types we have begun to characterize. Predominant emphasis in this thesis has been placed on data generated in BAEC and NRVM; however, future experiments in other cell types are planned to delineate the differences between cell types in the basal metabolic capacity.

**Defining Mitochondrial Respiratory State in vitro**

The presence of a reserve capacity, or spare respiratory capacity as mentioned above indicates that the cells are normally functioning at a sub-maximal capacity under basal conditions. An important goal of the studies described in this thesis was to relate parameters which are commonly used to describe mitochondrial function in isolated preparations to the actual measurement of mitochondrial function in cells. As discussed in the introductory chapter regarding the study of isolated mitochondria, mitochondria in maximal turnover with saturating substrates are said to be in State 3 respiration. Isolated mitochondria that are respiring on substrate, but without the presence of ADP, and thus without the proton import machinery of complex V are said to be in State 2 or 4 respiration. As has been suggested in other studies, it is unlikely that either of these extremes exist for mitochondria in cells; rather some intermediate turnover state, assigned in the literature as state 3.5 (the State\text{apparent}), likely prevails in the cellular context [100, 101].
While neither of these conditions may exist in cell culture they can be stimulated using the inhibitors described for the mitochondrial function assay above. In the next series of experiments, the assumption was made that State 3 respiration was equivalent to the rate measured after addition of FCCP. Similarly, State 4 was assumed to be the rate measured after addition of oligomycin, when the cells are incapable of utilizing the proton gradient to generate ATP due to the presence of the Complex V inhibitor (Figure 2-10). These assumptions allow for calculation of the apparent respiratory state of these cells under basal conditions, which is displayed in Figure 2-10 as State 3. For the purposes of this calculation, we will assume that the progression from State 3 to 4 is linear. The State_{apparent} can then be calculated from the following equation:

\[ \text{State}_{\text{apparent}} = 4 - \left( \frac{\text{Basal} - \text{Oligo}}{\text{FCCP} - \text{Oligo}} \right) \]

Eq. 2-1

where Basal represents the basal OCR, Oligo represents the Oligomycin-insensitive OCR, and FCCP represents the FCCP-stimulated OCR. This calculation yields an estimate of relative mitochondrial work being used by the cell under the basal condition. As ATP demand is increased, the cells approach an RCR of 3, or fully uncoupled respiration. When ATP/ADP ratios are high, and little mitochondrial turnover is required, the cells are expected to approach State 4. The State_{apparent} allows for an index of where cells fall on this scale following various interventions, as discussed below. From this calculation, an indication of mitochondrial workload can be inferred. Figure 2-11 displays the State_{apparent} for BAEC as a function of seeding density. Interestingly, the State_{apparent} appears to increase slightly with seeding density. This is likely due to the cells becoming
Figure 2-10: Cultured cells are in an intermediate respiratory state. A typical trace of OCR from an experiment using BAEC in the XF24 indicates that the basal respiration that is linked to ATP production is well beneath the maximal OCR achievable. The FCCP rate in this experiment is assumed to be equivalent to State 3 respiration. Similarly, the oligomycin-insensitive rate is equivalent to State 4. The basal respiration has thus been termed the State\textsubscript{apparent} and is defined as the proportional respiratory rate between States 3 and 4. Equation 2.1 demonstrates the calculation of this parameter.
Figure 2-11: Apparent respiratory state increases with cell density. Panel A: The State_{apparent} was calculated for BAEC seeded at 20,000–60,000 cells/well. Panel B: Basal RCR and Maximal RCR values were determined for the cells seeded at 40,000 cells/well. Data shown are the mean ± sem, n≥3 per group. *, p<0.05 vs. Basal RCR.
more quiescent with increased density. Thus mitochondrial workload is decreased, and
the \( \text{State} \text{apparent} \) approaches 4. An additional metric that can be used to determine the rela-
tive mitochondrial workload and efficiency is termed the Coupling Efficiency. This pa-
parameter describes the relative amount of oxygen consumption that is being used for ATP
generation as a function of the total basal oxygen consumption attributed to either ATP
production or proton leak. This parameter is calculated using the following equation:

\[
\text{Coupling Efficiency} = \frac{\text{Basal} - \text{Oligo}}{\text{Basal} - \text{Anti A}}
\]

Eq. 2-2

The data generated during the mitochondrial function assay can also be used to
calculate an apparent respiratory control ratio (RCR) for various metabolic conditions for
mitochondria in cells. Using the same assumptions regarding the relative State 3 and 4
respiration in cultured cells, the RCR is calculated as the State 3 rate minus the State 4
rate. The non-Cytochrome \( c \) Oxidase OCR (i.e. in the presence of antimycin) was sub-
tracted from all rates.

\[
\text{RCR}_{\text{basal}} = \frac{\text{Basal} - \text{Anti A}}{\text{Oligo} - \text{Anti A}}
\]

Eq. 2-3

\[
\text{RCR}_{\text{max}} = \frac{\text{FCCP} - \text{Anti A}}{\text{Oligo} - \text{Anti A}}
\]

Eq. 2-4

Figure 2-11 shows that the calculated RCR values are much larger than that typically de-
termined for isolated mitochondria. While it is unclear why this is the case, several ex-
planations can be postulated. First, the mitochondria in this experiment are not subject to any centrifugation during isolation, and are likely less prone to damage than the isolated mitochondria. Less membrane damage would result in decreased proton leak from the mitochondrial inner membrane. This would then lead to an increase in the RCR value measured as compared to that seen in isolated mitochondria. Additionally, we have not directly compared mitochondria isolated from any tissue to cells isolated from the same tissue concomitantly. While this information could serve as an interesting reference point between the two measurement techniques, the technical challenge that this approach requires precludes the ability to perform this calculation directly.

**Indication of real-time membrane permeability transition**

In order to demonstrate the use of optimal concentration of all compounds described above, dose response curves were generated. Interestingly, we have noted that oligomycin treatment at higher doses results in an increase in the OCR over time. To examine if this increase was due to a reversal of oligomycin inhibition of ATP synthase, an additional injection of oligomycin was performed in cells that exhibited a upwards trend in the OCR following an initial oligomycin treatment (Figure 2-12). BAEC were seeded to 40,000 cells per well and allowed to grow for 24 hours. Following three basal measurements of OCR, oligomycin was injected to 0.1, 0.3, 1, 3, or 10 μg/ml. An immediate inhibition of OCR was seen in all treatment groups (Figure 2-12 A, B). Cells treated with 3 μg/ml oligomycin or greater demonstrated an increase in OCR within the first three measurements of OCR following injection (second dotted line, Figure 2-12 C). An additional injection of oligomycin to double the concentration in the well demonstrates that
Figure 2-12: Oligomycin has disparate effects at different concentrations. BAEC were treated with the indicated concentration of oligomycin and monitored over time. Oligomycin should inhibit mitochondrial O₂ consumption to the degree to which it is linked to ATP production. Panel A demonstrates that there are differing effects of oligomycin at high concentrations. Oxygen consumption begins to increase, and this is not reversible with additional injection of oligomycin. The optimal concentration of this compound will have an immediate and maximal effect (Panel B, first dotted line in Panel A), and not stimulate O₂ consumption over time (Panel C, second dotted line in Panel A). All data shown are the means ± sem. n≥3 per treatment group.
this increase is not reversible by oligomycin, and indicates that the increase in OCR is not due to a lack of inhibition at ATP synthase.

**BENEFITS AND LIMITATIONS OF THE XF 24 METHOD**

While the Seahorse Bioscience XF24 has advanced our ability to assess cellular bioenergetics in multiple model systems, it is important to consider both the benefits and limitations of this analysis method in the interpretation of the data presented here. This section will discuss the perceived benefits and weigh them against the potential drawbacks in the context of the experimental models presented in Chapters 3-5 of this thesis. Importantly, the considerations discussed here may not be applicable in every scenario, and thus it is important to recognize that the use of the assays described in this chapter may not be optimal for all model systems.

**Throughput**

One strength of the XF technique is that multiple samples can be read simultaneously. This, coupled with the ability to multiplex data on oxygen and pH, allows for real-time determination of cellular bioenergetics across different treatment groups that was previously not possible. One of the closest existing experimental models to the mitochondrial function assay described in this chapter is the use of adherent cells in a flow system [98]. This model is comprised of two oxygen sensors, one placed upstream, and one downstream of cultured cells placed in a flow-cell. This apparatus allowed the authors to determine the real-time oxygen consumption in response to inhibitors, or compounds of interest, and also allowed for simultaneous detection of mitochondrial mem-
brane potential. While the ability to measure membrane potential in parallel is a large benefit to this type of experiment, only one sample can be measured at a given time. The authors of this paper report that a single experiment can take up to 200 min. Thus it is unlikely that more than 2 runs of this type of experiment could be performed in a given day. The ability to multiplex OCR and ECAR into a 24-well system using the XF24 now allows multiple complete experiments to be performed in a single day. This advance has increased the rapidity with which our understanding of whole cell bioenergetic responses has evolved.

Changes in Membrane Potential Occur With Treatment

One major limitation to the interpretation of the data presented in this thesis is that the mitochondrial membrane potential will change in response to the inhibitors used here. Importantly, there are differences in membrane polarization that occur between State 3 and State 4 respiration. The amount of proton leak occurring in a given system is largely controlled by the mitochondrial membrane potential. Because the magnitude of our measurements of proton leak and ATP-linked OCR will be dependent on the membrane potential, these parameters will necessarily be changed when any treatment is given that impacts on the membrane potential. In the set of studies described in the next chapters, oligomycin is used extensively to delineate the fraction of basal oxygen consumption that could be ascribed to ATP production at the ATP synthase, and proton leak across the membrane. Because oligomycin treatment induces a State 4-like respiratory condition, the mitochondrial membrane becomes hyperpolarized relative to the State 3 condition.
This translates to an overestimate of the Proton Leak parameter measured here due to the fact that the actual leak of protons will be higher after this treatment.

It has been suggested that this value could be corrected using known proton leak/membrane potential curves generated in isolated mitochondria. However, it is unlikely that this measurement can be corrected for in this manner without real-time parallel measurement of the mitochondrial membrane potential. This is partially due to the fact that the relative contribution of the proton leak to the total oxygen consumption at baseline is not linear during the progression from State 3 to State 4 respiration [103]. As expected, the proton leak plays an increasing role in the contribution to oxygen consumption as the cells approach State 4. Thus any attempt to base a correction factor for the proton leak measurement must not be made based on the assumed respiratory state at baseline, but instead on the measured membrane potential. The most accurate and sensitive method for performing this measurement is using tetramethylrhodamine methyl ester (TMRM). TMRM is a cationic dye that will accumulate according to the Nernst potential into the mitochondria [102]. Based on the relative fluorescence between the extracellular space, the cytosol, and the mitochondrial matrix, the mitochondrial membrane potential can be quantitatively determined [104-107]. This would then potentially allow for calibration to a known curve of the membrane potential/proton leak relationship. One further limitation of this method for correction lies in that the generation of a proton leak/membrane potential relationship curve requires isolated mitochondria. As discussed earlier in this chapter, there is likely some difference in the membrane integrity of isolated mitochondria as compared to those in the intact cell. This is suggested by the high
calculated $\text{RCR}_{\text{max}}$ values described in this chapter as compared to normal RCR values expected from isolated mitochondria. Thus it is possible that performing the corrective calculations will introduce a greater amount of error than our data currently contain.

Importantly, the data described in this thesis using the oligomycin-dependent measurements of proton leak and ATP-Linked OCR represent a large advance in our ability to measure these parameters \textit{in vitro}. It is then notable that the ability to compare the values generated between treatment groups is still able to provide important information regarding differences between those groups. Additionally, it is unlikely that the potential corrections discussed above would change the interpretation of the data. The major differences would lie in the absolute values reported for the Proton Leak and ATP-Linked measurements.

**Sample Size**

Another important benefit in the experiments described in subsequent chapters of this thesis is the small sample size. The XF 24 technique uses a very small number of cells in one experiment to perform the measurements described here. This fact thus allows for multiple analytical plates to be run, and allows parallel processing of other plates without using a large amount of starting material. This is especially important in studies such as that described in Chapter 4. The experiments described in that chapter utilize isolated, intact neonatal rat ventricular myocytes. These cells require rigorous attention to detail while isolating, and are only viable for 2-3 days following isolation. Furthermore, timed, pregnant dams must be ordered for specific arrival dates to allow for isolation to
be performed on 1-2 day old neonates. Thus the collection and experimentation on these
cells is both laborious and planning intensive. The ability to generate data from a small
amount of starting material is thus of increased importance.

**Sensitivity to Modifications of Cell Culture Technique**

One consideration that must be accounted for in the design of an XF Assay is the
cell culture technique. Because the XF 24 makes very sensitive measurements of a small
number of cells, it is imperative that these cells are cultured in a routine and reproducible
manner. During the course of the experiments described in this thesis, marked changes in
the response to both the compounds used for the mitochondrial function assay as well as
other interventions were noticed. The optimization of the cells lines discussed in Table
2-1 were typically performed with a 24 hour post-seeding analysis. Changes to this pro-
tocol result in large differences in the OCR and ECAR in nearly every cell line tested.
Importantly, departures from this protocol during experiments described in this thesis
were mentioned in the text. One example of the potential differences in the response of
the cells to various interventions was shown in Figure 2-11. This experiment described a
phenomenon whereby cells initially seeded at higher density had a calculated State$_{apparent}$
that approached 4. As discussed above, this likely indicates that the cells are more quies-
cent when seeded at a higher density. Such effects are likely cell type dependent, and
will thus vary with experimental model. Care should be taken to avoid these types of ar-
tifacts, and this is effectively done with strong attention to cell culture technique.
REAGENTS, EXPERIMENTAL DESIGN, AND CELL CULTURE

Reagents

Specialized tissue culture plates, hydration solution, and assay cartridges for the Extracellular Flux assays were purchased from Seahorse Biosciences (North Billerica, MA) as FluxPaks. Antimycin A, carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP), nonanal, oligomycin, potassium cyanide, rotenone, thenoyl trifluoroacetone (TTFA), and thiazoyl blue tetrazolium were from Sigma (St. Louis, MO) and of the highest grade offered. \((Z)-1-[2-(2-Aminoethyl)N-(2-ammonioethyl)amino]diazen-1-iium-1,2-diolate\) (DetaNONOate) was from Cayman Chemical (Ann Arbor, MI). 2,3-dimethoxy-1,4-napthoquinone (DMNQ) was obtained from Alexis Biochemicals (San Diego, CA). Reagent 4-hydroxy-2-nonenal (HNE) was from Calbiochem (San Diego, CA).

Antibodies and Western Blotting Materials

Anti-protein HNE antibodies were a gift from Dr. Sanjay Srivastava at the University of Louisville. Control IgG (Cell Signaling Technologies, Danvers, MA) was used as a control in some experiments. Secondary anti-rabbit HRP-linked antibody for detection of Protein-HNE adducts and anti-\(\beta\)-actin antibody were also from Cell Signaling. ECL plus reagents used in the development of HNE immunoblots were from GE Healthcare (Pittsburgh, PA). Antibodies directed against NADH-ubiquinone oxidoreductase (Complex I) 39kDa Subunit, Ubiquinone Cytochrome c Oxidoreductase (Complex III) Core I, and Cytochrome c Oxidase (Complex IV) Subunits I, IV, and Vb were from
Invitrogen (Carlsbad, CA). Antibodies for cleaved Caspase 3 and Procaspsase 9 were from Cell Signaling Technologies.

Relative protein levels were quantified using SDS-PAGE and Western blotting for proteins of interest. For experiments in BAEC or MDA-MB231, cells were washed twice with PBS and lysed with a lysis buffer containing 10mM Tris pH 7.4, and 1% Triton-X 100. Protein concentration in the whole cell lysate was determined by the Bradford method. Whole protein samples were loaded onto 12.5% SDS-PAGE gels for resolution before transfer to PVDF membranes. Secondary antibody detection for Western blots shown in Chapters 5 and 6 was performed using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Rockford, IL). Blots were imaged using an Alpha Innotech cooled-CCD imaging system using the included Alfa Ease software (San Leandro, CA). For experiments in NRVM, cells were lysed in buffer containing 20 mM HEPES, 1 mM DTPA, 1% NP-40, 0.1% SDS, and protease inhibitor cocktail. The lysates were then added to SDS Laemmli Buffer, and the proteins were separated on 10% SDS polyacrylamide gels. Proteins were transferred to PVDF membranes, and HNE-modified proteins were detected using anti-protein-HNE primary and HRP-linked antirabbit secondary antibodies by chemifluorescence on a Typhoon Variable Mode Imager (GE Healthcare, Pittsburgh, PA).

**Bovine Aortic Endothelial Cell Culture**

Bovine aortic endothelial cells (BAEC) were harvested from descending thoracic aortas and maintained at 37°C with 5% CO₂ in Dulbecco’s Modification of Eagle’s Me-
dium (DMEM) growth medium (Mediatech, Manassas, VA) supplemented with 5.5 mM D-Glucose (Sigma, St. Louis, MO) 4 mM glutamine, 1 mM pyruvate, 3.7 g/L sodium bicarbonate 100 U/ml penicillin and 100 ng/ml streptomycin all from Invitrogen (Carlsbad, CA) and 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA). Cells used in this study were between passages 5–8. A representative photomicrograph of these cells is shown in Figure 2-13.

**Neonatal Rat Ventricular Myocyte Primary Cultures**

All animal experiments were approved by the University of Alabama Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Primary cultures of neonatal rat ventricular myocytes (NRVM) were obtained from 2- to 3-day-old neonatal Sprague-Dawley rats and were cultured as described previously [108]. NRVM were seeded at 75,000 cells/well in collagen-coated Seahorse Bioscience V7 culture plates in growth medium containing 15% fetal bovine serum (FBS) on the first day. On the next day, medium was replaced, and cells were grown in the culture growth medium without FBS. Within 1–2 days of isolation, a confluent monolayer of spontaneously beating NRVM formed, and cells were used as described below. A representative photomicrograph of these cells is shown in Figure 2-13.

**MDA-MB-231 Cell Culture**

MDA-MB231 human mammary adenocarcinoma cells were cultured in RPMI 1640 media (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum.
**Figure 2-13: Images of cell types used in this thesis.** Bovine aortic endothelial cells are displayed in a 20x field to the left, and primary neonatal rat ventricular myocytes are shown at 10x on the right. These images demonstrate the heterogeneity of the cell types used in this study, and highlight the need for thorough characterization of each cell line used in this technique, as there can be significant differences in morphology and growth patterns between cell types.
(FBS; Atlanta Biologicals, Atlanta, GA). Cultures were maintained in 5% CO2 and humidified in a 37°C incubator. All experiments were performed at 0.5% FBS in RPMI 1640 media at ~50% confluence.

**HUVEC Cell Culture**

Primary Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in EBM media (Lonza, Walkersfield, MA) and maintained at 37°C with 5% CO2 in a humidified atmosphere. The media was supplemented with an EGM Singlequot consisting of Bovine Brain Extract, human EGF, Hydrocortisone, GA-1000, and 2% FBS. Cells used in this study were of passage 2-3.

**CELL VIABILITY**

Cell viability was measured by MTT assay as described previously [109], with the following modifications. BAEC were seeded as described above into 48-well culture plates at 100,000 cells/well. After 6 h, the assay media was replaced with media containing 0.4 mg/ml thiazoyl blue tetrazolium (Sigma, St. Louis, MO). The cells were allowed to incubate in a non-CO2 incubator at 37°C for an additional 2 h. The media was removed, and the resulting formazan crystals were solubilized in 250 μl DMSO. The absorbance was read at 550 nm and the data expressed as the percent viability of control cells.
PROTEIN-HNE AND GSH MEASUREMENTS

Protein-HNE adducts were detected using a polyclonal anti-protein HNE antibody, as described in [84]. Briefly, cells were lysed in the 24-well XF plates using 20 μl/well of lysis buffer containing 20 mM HEPES, 1 mM DTPA, 1% NP-40, 0.1% SDS, and protease inhibitor cocktail. The lysates were then added to SDS Laemmli Buffer, and the proteins were separated on 10% SDS polyacrylamide gels. After transfer to PVDF membranes, HNE-modified proteins were detected using anti-protein-HNE primary and HRP-linked anti-rabbit secondary antibodies by chemifluorescence on a Typhoon Variable Mode Imager (GE Healthcare, Pittsburgh, PA). Relative levels of protein-HNE adducts were quantified by densitometry using ImageQuantTL software (GE Healthcare, Pittsburgh, PA). Total GSH (GSH+GSSG) was measured in cell lysates with and without treatment with HNE using the Tietze recycling assay [110].

FLUORESCENCE MICROSCOPY

All fluorescence microscopy data in this thesis were collected using microscopes and equipment maintained by the UAB High Resolution Imaging Facility. All images are confocal micrographs acquired using the listed excitation and emission settings on a Leica DMIRBE Laser Scanning Confocal Microscope. This microscope allows for multiple fluorophore detection on up to three separate channels using three separate photomultiplier tubes. This allows for highly sensitive image acquisition with little interference from other fluorophores present in the sample. Live images were averaged 4 times by the Leica TCS software, and saved as 8-bit TIFF images to compact disc. The files
were then transferred to a personal computer in our lab for processing and manipulation as described below.

**Subcellular Localization of HNE**

To determine the subcellular localization of HNE-protein adducts exposed to HNE, NRVM grown on borosilicate glass coverslips were fixed with 3.7% formaldehyde (Tousimis, Rockville, MD) in PBS for 10 min. The cells were then permeabilized with 0.1% Trixon X-100 in PBS for 5 min. Coverslips were then incubated in 10% goat serum (Vector Labs, Burlingame, CA) for 1 h at room temperature. HNE adducts were then probed using a 1:100 dilution of Anti-HNE antibody. Following overnight incubation with this antibody in 10% goat serum containing PBS, the cells were washed three times with PBS for 5 min each time to remove any unbound primary antibody. Fluorescent secondary antibody (1:1000 Chicken anti-rabbit AF488 to detect HNE) was used to detect the primary antibody. The coverslips were then be mounted in DAPI containing medium (Vector Labs, Burlingame, CA), and imaged using confocal laser scanning microscopy. HNE signal was determined using excitation from a 488nm Argon laser line and emission detection suitable for fluorescein. Nuclear localization of DAPI was confirmed using excitation from a UV laser with emission detection suitable for this fluorophore.

**F-Actin Visualization for Cytoskeletal Morphology**

To determine the extent of actin polymerization, treated cells grown on glass coverslips were fixed with 3.7% formaldehyde (Tousimis, Rockville, MD) for 10 min. The cells were then washed twice with PBS and incubated for 5 min at room temperature with
0.5 % Triton X-100 in PBS. Next, cells were incubated with blocking solution (PBS containing 1% BSA) for 30 min followed by application of 2 units of Texas Red X-phalloidin (Invitrogen, Carlsbad, CA) in blocking solution for 30 minutes at room temperature. The coverslips were then mounted on glass slides using a DAPI-containing mounting medium (Vector Labs, Burlingame, CA). F-actin imaging was performed using laser scanning confocal microscopy with a 561nm laser line for excitation, and emission detection suitable for Texas Red. Nuclei were imaged using excitation from a UV laser, and emission detection suitable for DAPI. Images were merged and processed using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

**SIRNA KNOCKDOWN OF NUCLEAR-ENCODED MITOCHONDRIAL PROTEINS**

All siRNA constructs used in this study are commercially available for the human gene sequence, and are directed at specific subunits of cytochrome c oxidase. HUVEC grown in 6-well plates were treated with the siRNA construct complexed to Lipofectamine 2000 at a final concentration of 20 μM. All transfections were done in antibiotic free OptiMEM media (Gibco; Carlsbad, CA), and performed for eight hours. After eight hours, the media was changed and the cells were allowed to grow for an additional 40 hours. To confirm knockdown of each targeted gene, commercially available monoclonal antibodies (Molecular Probes, Invitrogen; Carlsbad, CA) were used to detect functional protein by SDS-PAGE and Western blot analysis.
CHEMICAL STRUCTURES

All chemical structures illustrated in this thesis were created using ACD ChemSketch 11.01 (Advanced Chemistry Development Inc., Toronto, Ontario, Canada). After rendering, structures were exported as TIFFs. These images were processed using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

DATA ANALYSIS AND STATISTICAL TESTS

All data from XF Assays described in this thesis were collected using the XF Reader software (version 1.3.0.62 or 1.4.1.29) from Seahorse Biosciences (North Billerica, MA). Data from these experiments were exported to Microsoft Excel 2007 (Microsoft Corp., Redmond, WA), and analyzed, transformed, and charted there. Statistical significance was determined by performing 2-tailed Student’s t-tests where appropriate using Excel 2007, or by One-Way ANOVA using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) as indicated. \( p \leq 0.05 \) was considered significant in all experiments.

Data from Western blots in Chapters 5 and 6 were obtained using AlfaEase software (Alpha Innotech, San Leandro, CA), and exported into Microsoft Excel 2007. Data from Western blots of protein-HNE adducts in Chapter 4 were quantified by densitometry using ImageQuantTL software (GE Healthcare, Pittsburgh, PA), and exported into Microsoft Excel 2007. Intensity values are plotted in Excel, and statistical significance determined by 2-tailed Student’s t-tests where appropriate using Excel 2007. \( p \leq 0.05 \) was considered significant.
The fluorescence intensity of all microscopy images generated was quantified using SimplePCI Software (Compix, Cranberry Township, PA). The intensity of the staining of each treatment was expressed as a fold increase as compared to the control treatment. Manipulation and merging of color channels was performed using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).
CHAPTER 3
MITOCHONDRIAL RESERVE CAPACITY IN ENDOTHELIAL CELLS: THE IMPACT OF ACUTE NITRIC OXIDE AND REACTIVE OXYGEN SPECIES

INTRODUCTION

In the first series of experiments designed to utilize the techniques outlined in Chapter 2, the effects of known physiological modulators of endothelial cell function were examined. Importantly, production of nitric oxide (NO’) by endothelial cells to cause vasorelaxation was demonstrated more than 20 years ago [111, 112]. More recently, the understanding that NO’ production impacts on vascular function beyond its role as a vasodilator has been described [78, 113]. However, specific studies of the impact of NO’ on endothelial bioenergetics are lacking in the literature. This is largely due to a relative paucity of mitochondria in these cells, making traditional isolated mitochondria studies difficult. NO’ has been proposed to modulate endothelial respiratory function due to the persistent production of NO’ from eNOS, and also through the known inhibition of Cytochrome c Oxidase by this molecule [77, 114, 115]. The set of studies described in this chapter demonstrate that NO’ can impact strongly on endothelial function, and confirm the role of the reserve capacity in the function and survival of these cells in response to oxidative stress.
Nitric Oxide and the Endothelium

The endothelium maintains vascular function and is exquisitely sensitive to reactive oxygen and nitrogen species (ROS/RNS) [112, 116]. NO’ is particularly important for cardiovascular health and plays both protective and maladaptive roles in vascular diseases associated with oxidative stress. For example, at low concentrations NO’ has been shown to protect against cell death induced by hydrogen peroxide but at higher concentrations to enhance the toxicity of ROS [29, 30, 117, 118]. The injurious role of NO’ in disease has been attributed primarily to biochemical interactions with superoxide (O₂⁻) to form peroxynitrite (ONOO’), a highly oxidizing species [119, 120]. In the endothelium, NO’ is derived from endothelial or inducible nitric oxide synthases, and O₂⁻ is derived from oxidase enzymes or the mitochondrial electron transport chain [121, 122]. Interestingly, the concentration and temporal coordination of exposure to these reactive species appear to be particularly important for cell signaling, metabolism, and the commitment of the cell to survival or death [123].

Researchers have attempted to examine the role of NO’ in regulation of endothelial cell function. Measurements of oxygen consumption in response to NO’ have been performed in stirred non-adherent endothelial cells [124] and fibroblasts [75], as well as in stirred cells grown on microcarrier beads [76]. These and other studies have shown that NO’ plays a significant role in Complex IV inhibition at low oxygen concentrations [125], and also has an inhibitory effect on oxygen consumption at normoxia [76]. Nevertheless, the detailed molecular mechanisms by which NO’ and other reactive species control mitochondrial function in endothelial cells remain unclear.
Mitochondria and Nitric Oxide in Cardiovascular Disease

Mitochondria are of particular interest because they are known to be damaged during the progression of atherosclerosis [126-128]. This damage appears to be ROS/RNS-dependent and causes loss of bioenergetic control which leads to vascular dysfunction. In most studies examining the role of mitochondria in cardiovascular disease, the experimental approach has been to study mitochondria isolated from the heart and assess the impact of ROS/RNS on established mitochondrial bioenergetic parameters such as oxygen consumption and respiratory control. Such studies show that interaction of NO’ with mitochondria is reversible and occurs primarily at cytochrome $c$ oxidase [64, 129], is competitive with oxygen [73, 130, 131], and is more effective as an inhibitor of oxygen consumption when mitochondria are in state 3 as compared to state 4 respiration [130, 132]. While polarographic techniques have been a valuable tool for studies in isolated mitochondria, measuring bioenergetic function in intact cells using this methodology has proved more challenging. As discussed in Chapter 2, there are several major disadvantages of polarographic measurements in that the cells must be both continuously stirred and free from matrix attachment. In endothelial cells, this detached state may result in anoikis which is associated with increased ROS and mitochondrial damage [133]. In addition, non-laminar shear, which occurs as a result of stirring in the oxygen electrode, will result in increased oxidative stress [134, 135]. Researchers have attempted to partially overcome this by seeding cells on micro-carrier beads to prevent anoikis, however these cells are still subject to non-laminar shear [76]. Thus an understanding of how endothelial mitochondria respond to NO’ and reactive species in a cellular setting is incomplete.
In this series of experiments, the effects of NO’ and ROS on cellular bioenergetic function in adherent bovine aortic endothelial cells (BAEC) were examined. Additionally, mitochondrial respiratory control in the cells was evaluated by calculating an apparent respiratory state, analogous to the parameters of State 3 and 4 which are frequently determined with isolated mitochondria. This allows for a more direct comparison to studies with isolated mitochondria and is particularly useful for endothelial cells where it is not possible to prepare the large quantities of mitochondria needed for polarographic measurements. Using this approach, the data in this chapter demonstrate how a basal apparent respiratory control ratio can be determined in the absence or presence of stressors such as ROS/RNS in intact cells. Lastly, the concept of mitochondrial reserve capacity in cells is discussed, with an analysis of the effects of redox stressors on this bioenergetic parameter.

RESULTS AND DISCUSSION

Impact of NO’ on Bioenergetic Parameters in Endothelial Cells

In the first series of experiments, we examined the effects of NO’ and ROS on cellular bioenergetic function in adherent bovine aortic endothelial cells (BAEC). Using the extracellular flux technology described in Chapter 2, the bioenergetic state of these cells was characterized and basal mitochondrial function was determined. As mentioned above, NO’ is an important physiologically relevant modulator of mitochondrial respiration [136]. We and others have shown in isolated mitochondria that NO’-dependent inhibition of respiration at Cytochrome c Oxidase is far greater in State 3 than State 4 [130,
Figure 3-1: Acute nitric oxide treatment decreases reserve capacity, but has no effect on baseline oxygen consumption. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. After a basal measurement of OCR, cells were treated without or with DetaNONOate (0–500 μM) for 1 h. This figure shows representative traces for 0, 250, and 500 μM DetaNONOate. All cells were then treated sequentially with 1 μg/ml oligomycin, 1 μM FCCP and 10 μM antimycin A. Oxygen consumption was measured after each injection, and is displayed as the percentage of baseline for each treatment group. Data shown are the means ± sem, n≥3 per group.
These data suggest that inhibition of mitochondrial respiration in cells will be highly sensitive to the turnover of (electron flux through) the respiratory chain. The effect of the apparent intermediate respiratory state occurring in cells, however, has not been examined. Therefore, we determined the impact of NO' on the basal OCR using controlled generation of exogenous NO' from NO' donors. Adherent BAEC were treated with DetaNONOate (0–500 μM) for a period of 1 h prior to measurement of mitochondrial function as described in Chapter 2. The levels of NO' released by Deta NONOate under these conditions were estimated to be in a similar range to that reported for inducible nitric oxide synthase (0-5 fmols per min per cell) [137, 138].

As shown in Figure 3-1, injection of Deta NONOate into the experimental well caused no significant change in the basal OCR even after 1 h incubation. This figure shows only the 250 and 500 μM treatment groups for clarity, however this effect was seen at all concentrations tested from 50-500 μM Deta NONOate. The third measurement following incubation with the NO' donor was considered the baseline OCR following NO treatment and is shown in Figure 3-2 Panel A. The absence of change in the basal OCR is consistent with a lower sensitivity of the respiratory chain to NO' when in a low state of turnover. This would be the case in a State-4-like respiration in an analogous isolated mitochondria experiment, however endothelial cells are not normally in State 4 respiration as demonstrated in Chapter 2. Upon addition of FCCP to stimulate the equivalent of State 3 respiration, OCR was inhibited in an NO'-dependent manner (Figure 3-1, 3-2 Panel B). Since NO'-dependent inhibition of cytochrome c oxidase is known to increase with decreasing oxygen concentration [73, 139], we next determined whether the
Figure 3-2: Acute nitric oxide treatment decreases reserve capacity, but has no effect on baseline oxygen consumption. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. Panel A: Effect of acute NO treatment on basal mitochondrial function. The third rate taken post-NO injection (Shown in Figure 3-1) is plotted as a function of Deta NONOate concentration. No points are statistically significant vs. CTL. Panel B: The change in FCCP-stimulated OCR is shown as a percentage increase from the original baseline and a function of Deta NONOate concentration. Panel C: Oxygen consumption as a result of mitochondrial activity was plotted in the absence (■) or presence (□) of 500 μM Deta NONOate. Data shown are the measured oxygen concentrations during the OCR measurement taken immediately following FCCP injection. Panel D: Mitochondrial reserve capacity in cells treated with Deta NONOate. Data shown are the means ± sem, n≥3 per group.
oxygen concentration was the same for all cells before the addition of NO’, as well as at the beginning of each measurement of oxygen consumption. For example, as shown in Figure 3-2 Panel C, inhibition of the FCCP-stimulated rate by NO’ was evident at the first O2 measurement after FCCP addition and persisted over the entire time of measurement. The consequence of the effect of NO’ on the FCCP stimulated rate was an approximately 4-fold decrease in the reserve respiratory capacity from 94.5 ± 16.5 to 24.6 ± 4.2 pmol O2/min (Figure 3-2 Panel D). Importantly, endothelial cells are exposed to high concentrations of NO’ as they specifically generate this compound for vasorelaxation of the arterial wall. These data suggest that NO’ may have little effect on endothelial cells under the basal condition, and that only under conditions of a second-hit stress is a significant inhibition of OCR observed. This concept will be discussed in greater detail below.

Because the effects of NO’ are known to differ with respiratory state, we next calculated the apparent respiratory state in these cells in response to NO’ treatment. Interestingly, the calculated Stateapparent decreased from 3.67 ± 0.04 to 3.32 ± 0.03 in cells treated with 500 μM Deta NONOate. Because State 3 respiration in this analysis is considered to be the maximal respiratory rate, these data indicate that the cells approached their maximal respiratory capacity after the NO’ treatment, (Figure 3-3). This effect is a direct result of the decreased OCR stimulated by FCCP. We found this effect to be concentration dependent with NO’, but it appears to be nearing a maximal effect with 500μM Deta NONOate.
Figure 3-3: Acute nitric oxide decreases $\text{State}_{\text{apparent}}$. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. Deta NONOate was injected at the indicated concentrations, and the cells were allowed to incubate for 1 h. The $\text{State}_{\text{apparent}}$ was calculated using the third post-NO’ injection rate as the basal rate (Shown in Figure 3-1). The data are plotted as a function of Deta NONOate concentration. Data shown are the means ± sem, $n \geq 3$ per group.
Additionally, the effect of acute NO’ treatment on ECAR was determined and calculated as the change in mpH/min (Figure 3-4). In contrast to what was seen with OCR, we found a dose-dependent increase in the basal ECAR levels. This indicates a stimulation of pathways that acidify the media in response to NO’. It has been suggested that glycolysis is the predominant mechanism of extracellular acidification due to the release of lactate [60]. In concordance with the OCR data, the stimulation of ECAR seems to be maximal nearing 500 μM Deta NONOate. Interestingly, oligomycin is able to stimulate the ECAR to a still higher rate. This indicates that the NO’ treatment is not stimulating a maximal increase in glycolysis, but is instead saturating an intermediate signaling pathway. While the mechanism of this induction is still unknown, there are several suggestions from the literature that show promise. For example, AMP-activated protein kinase (AMPK) has been shown to be sensitive to NO’ and cause upregulation of glycolysis in iNOS-transfected astrocytes [140, 141]. This suggests that AMPK is sensitive to NO’-mediated changes in cellular bioenergetic status. The measurement of ECAR also allows for determination of the metabolic profile of BAEC in response to acute exposure to NO’ as shown in Figure 3-4 Panel B. This graph supports the concept that NO’ generated from Deta NONOate has little effect on the basal OCR, but increases ECAR and thus shifts the cells to a higher energy producing state than the control cells.

Taken together these data extend the findings with isolated mitochondria that the turnover of the respiratory chain is an important determinant in defining the sensitivity to inhibition by NO’. A limitation of the present study inherent in the design of the XF24 analyzer is that the experiments were performed at ambient oxygen concentrations which
Figure 3-4: Acute nitric oxide increases the extracellular acidification rate. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. Deta NONOate was injected at the indicated concentrations, and the cells were allowed to incubate for 1 h. The basal ECAR was determined for each treatment group and is plotted as a function of Deta NONOate concentration (Panel A). The net effect on the metabolic profile was determined by plotting OCR (Shown in Figure 3-2 A) vs. ECAR for each Deta NONOate concentration (Panel B). Data shown are the means ± sem, n≥3 per group. *, p<0.05 vs. Control.
potentially decreases the ability of NO\textsuperscript{−} to inhibit the respiratory chain [73, 130]. This is because NO\textsuperscript{−} competes with oxygen at the active site of Cytochrome \textit{c} Oxidase, and also is consumed by the mitochondrial inner membrane [79, 80]. However, since the oxygen levels are several orders of magnitude above the concentrations needed to saturate Cytochrome \textit{c} Oxidase, these higher oxygen concentrations are unlikely to impact on the State\textsubscript{apparent} of the mitochondria. Additionally, since at lower oxygen levels the autoxidation of NO\textsuperscript{−} will be decreased, the NO\textsuperscript{−} available to inhibit Cytochrome \textit{c} Oxidase will increase [80, 142]. It is therefore predicted that the NO\textsuperscript{−}-dependent decrease in reserve capacity will be enhanced at lower oxygen tensions.

**The Effect of Endogenous Nitric Oxide on Mitochondrial Function in BAEC**

The above described studies were performed in cells treated with continuous, sustained release of NO\textsuperscript{−} by an exogenously administered nitric oxide donor (Deta NONOate). In order to determine if the same effects on cellular bioenergetic function could be observed with endogenous production of NO\textsuperscript{−}, we next performed a series of experiments utilizing Bradykinin (Bk) and Acetylcholine (ACh). Bradykinin is a small-molecule peptide that binds to the Bradykinin receptor on endothelial cells. Binding to this receptor causes an influx of calcium into the cell from the extracellular space and raises the intracellular calcium level. Importantly, the endothelial nitric oxide synthase (eNOS) requires Ca\textsuperscript{2+} as a cofactor in the production of NO\textsuperscript{−} from arginine. Bradykinin has been shown to increase NO\textsuperscript{−} generation in BAEC, and we examined the effect of this endogenously produced NO\textsuperscript{−} in the next series of experiments. Similarly, ACh has been shown to increase NO\textsuperscript{−} production in an endothelium-dependent manner [111, 143]. In separate experi-
Figure 3-5: Bradykinin and acetylcholine inhibit FCCP-mediated induction of oxygen consumption, but not basal OCR. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. After a basal measurement of OCR, cells were treated without or with Bradykinin (0–10 μM) (Panel A), or Acetylcholine (0-100μM) (Panel B) for 1 h. Three OCR measurements were then made, and the final measurement for each treatment is shown (□). All cells were then treated sequentially with 1 μg/ml oligomycin, 1 μM FCCP and 10 μM antimycin A. Oxygen consumption was measured after each injection, and the FCCP rate is displayed as a percentage of baseline for each treatment group (■). Data shown are the means ± sem, n≥3 per group.
ments, both Bk and ACh demonstrated no effect on basal OCR in BAEC (Figure 3-5). Interestingly, only in the presence of FCCP is there any inhibition seen with either of these compounds. Filled squares demonstrate the response to FCCP in the presence of the indicated concentration of Bk or ACh. As shown in this figure, stimulation of the OCR by FCCP is inhibited by both Bk and ACh in a dose-dependent manner.

**The Effect of ROS Production on Mitochondrial Reserve Capacity in BAEC**

Next, we examined the effects of acute oxidative stress on mitochondrial bioenergetics by exposing the cells to the redox cycling agent 2,3-dimethoxy-1,4-napthoquinone (DMNQ, 15 μM) for 1 h (Figure 3-6). DMNQ enters endothelial cells and generates both superoxide and hydrogen peroxide at a rate depending upon its concentration and similar to that generated by enzymes such as NADPH oxidase [144]. We have previously shown that this concentration will induce cell death through an apoptotic process in BAEC if exposure proceeds longer that 3–4 h [29]. As shown in Figure 3-6, DMNQ stimulated the basal OCR, and this stimulation was independent of the addition of mitochondrial inhibitors, occurring even in the presence of antimycin A. This indicates that the increase in oxygen consumption is due to the redox cycle of DMNQ and not a stimulation of the mitochondrial electron transport chain. Under these conditions, DMNQ alone has a minimal effect on proton leak (Figure 3-7 Panel A). Importantly, DMNQ inhibited the FCCP-induced oxygen consumption in a concentration-dependent manner resulting in a decrease in the reserve capacity (Figure 3-7 Panel B). This led to a decrease in the State*apparent* as the cells lost their maximal respiratory capacity (Figure 3-7 Panel C).
Figure 3-6: Acute DMNQ treatment decreases maximal OCR and increases basal OCR. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. Panel A: Three baseline measurements of OCR and ECAR were made, and then cells were treated with DMNQ (15 μM) for 1 h. Control-treated cells are represented by the filled squares (■), and DMNQ-treated cells are represented by the open squares (□). Following this treatment, three further measurements were performed prior to sequential injection of oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) to determine mitochondrial function. Panel B: The increase in basal OCR due to DMNQ (0-15 μM) was determined and is plotted as a function of DMNQ concentration. Data shown are the means ± sem, n≥3 per group. *, p<0.05 vs. Control.
**Figure 3-7: DMNQ decreases reserve capacity and respiratory State\textsuperscript{apparent}.** Panel A: Proton leak was calculated and is shown for control and DMNQ (15 μM)-treated cells. Panel B: Reserve capacity following DMNQ treatment is also plotted as a function of DMNQ concentration. Panel C: Apparent respiratory state was calculated as described and is shown for control and DMNQ (15 μM)-treated cells. Panel D: Diphenyleneiodonium (10 μM) was added with the DMNQ in some treatment groups. Inhibition of DMNQ-dependent OCR stimulation is shown. Data shown are the means ± sem, n≥3 per group. *, p<0.05 vs. Control unless otherwise indicated.
As a control for the requirement of redox cycling for DMNQ-stimulated oxygen consumption, the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 μM) was added and found to prevent the DMNQ-dependent increase in basal OCR (Figure 3-7 Panel D). Interestingly, DPI itself inhibited the induction of the OCR due to FCCP treatment (data not shown). Because Complex I contains several flavoproteins for electron transfer, this data is consistent with a normal stimulation of electron flux through complex I during maximal turnover stimulated by FCCP. No significant change in the basal ECAR was detected under any of the treatment conditions with DMNQ (data not shown).

The data presented so far in this chapter suggest that treatment of cells in culture with exogenously-generated NO$^\cdot$ has little effect on basal oxygen consumption. As mentioned above, endothelial cells lining blood vessels may only be impacted by the large amounts of NO$^\cdot$ that they produce when exposed to a secondary stressor, such as that which may occur during the progression of atherosclerosis [29, 30]. The next series of experiments was designed to examine this concept.

**The Combined Effect of NO$^\cdot$ and DMNQ on Mitochondrial Function in BAEC**

In the previous experiments we have demonstrated that both DMNQ and NO$^\cdot$ are capable of decreasing the mitochondrial reserve capacity; however, these species by themselves do not impact significantly on the basal OCR. Under conditions of inflammation, endothelial cells are exposed to both NO$^\cdot$ and ROS, and this was modeled in the next series of experiments. As shown in Figure 3-8 Panel A, the cells were exposed to a combination of 250 μM Deta NONOate and 15 μM DMNQ for 60 min, and mitochondri-
Figure 3-8: Proton leak is stimulated by combined Deta NONOate and DMNQ treatment. BAEC were seeded at 40,000 cells/well and allowed to grow for 24 h. Three baseline measurements of OCR and ECAR were made and then cells were treated with Deta NONOate (250 μM) and DMNQ (15 μM) combined for 1 h. Control-treated cells are represented by the filled squares (■), and Deta NO + DMNQ-treated cells are represented by the open squares (□). Following this treatment, three further measurements were performed prior to sequential injection of oligomycin (1μg/ml), FCCP (1μM), and antimycin A (10 μM) to determine mitochondrial function. Data shown are the means ± sem, n≥3 per group. For visual clarity, statistical indicators are omitted from this graph. More detailed analysis follows in Figure 3-9.
Figure 3-9: Proton leak but not non-mitochondrial OCR is stimulated by combined Deta NONOate and DMNQ treatment. Panel A: Proton leak was calculated as the oligomycin-insensitive OCR minus the antimycin A-insensitive OCR. The non-mitochondrial, or antimycin A-insensitive OCR was also determined (Panel B). Data shown are the mean ± sem, n≥3 per group. *, p<0.05 vs. Control.
al function was analyzed. This resulted in a dramatic suppression of mitochondrial function associated with an increase in oligomycin-insensitive oxygen consumption (Figure 3-8, 3-9 Panel A). In addition, there was a significant loss of responsiveness to FCCP after these treatments. Importantly, these effects on mitochondrial function do not appear to be due to cellular production of ROS, as the increase in the antimycin A-insensitive OCR is only moderate, and did not reach statistical significance (Figure 3-9 Panel B).

As mentioned above, NO\(^{-}\) is known to increase the toxicity of ROS in several model systems [29]. To determine the long term effects of this treatment regimen on cells, we next examined cell viability using the MTT Assay. As shown in Figure 3-10, BAEC were treated with the indicated concentrations of DMNQ and/or Deta NONOate for 6 h. DMNQ decreased cell survival at concentrations above 7.5 \(\mu M\) whereas the Deta NONOate (500 \(\mu M\)) had a small but significant effect in the absence of DMNQ. In the presence of both the NO\(^{-}\) donor and DMNQ, however, toxicity was greatly increased with as little as 5 \(\mu M\) DMNQ when in combination with the NO\(^{-}\) donor. This suggests that NO\(^{-}\) has a significant effect on endothelial cell function that serves to increase the sensitivity to ROS. This sensitization is important in relation to the pathogenesis of atherosclerosis, where increased ROS and NO\(^{-}\) are thought to contribute to endothelial dysfunction. Proper endothelial function is crucial to maintaining vascular compliance and preventing neointimal formation following vessel repair. Further studies will examine a possible role for stimulation of bioenergetic reserve capacity as a protective mechanism against the increased cell death seen with this treatment.
Figure 3-10: Combined Deta NONOate and DMNQ treatment results in greater cytotoxicity than either treatment alone. Cell viability was determined using the MTT assay for cells treated with 500 μM Deta NONOate and the indicated concentration of DMNQ as a cotreatment for 8 h. Cell viability was calculated as a percentage of the control treated cells. Data shown are the means ± sem. #, p<0.05 vs. matched concentration DMNQ-only control.
SUMMARY

In this study, we have demonstrated the development of an assay for determination of mitochondrial function in intact endothelial cells. These experiments show that BAEC have basal O$_2$ consumption rates that are only ~35% of the maximal oxygen consumption achievable using the uncoupler FCCP. This indicates the presence of a reserve capacity or spare respiratory capacity that is available for the cells to call upon when bioenergetic demand is increased. BAEC in culture normally consume oxygen in an intermediate State between State 3 and 4 [145, 146], which we calculate to be approximately State$_{apparent}$ 3.67. It is of note that the calculated RCR values for these cells indicate that the mitochondria are tightly coupled under normal physiological conditions. Importantly, this indicates that the endothelium in vivo may be well poised to offer protection against oxidative and nitrosative stress that typically occurs in the vasculature during the pathological processes associated with vascular dysfunction. Furthermore, these data suggest that modulating the bioenergetic status of these cells may be a useful method for preventing endothelial dysfunction and death. This could offer benefit by slowing the development of atherosclerosis and other vascular diseases.
CHAPTER 4

IMPORTANCE OF THE BIOENERGETIC RESERVE CAPACITY IN RESPONSE TO CARDIOMYOCYTE STRESS INDUCED BY 4-HYDROXYNONENAL

INTRODUCTION

In the previous chapter, data was presented demonstrating both the presence of a reserve capacity in cultured cells, and also that this reserve capacity can be depleted in response to acute treatment with nitric oxide. Importantly, this data demonstrated depletion of the reserve capacity in spite of having little impact on the basal oxygen consumption rate (OCR), but does not address a mechanism whereby the cells may increase utilization of this respiratory parameter. This concept has proved technically difficult to examine using direct indices of mitochondrial function in intact cultured cells due to the inability to measure these cells using standard respiratory techniques. As discussed in Chapter 2, advances in extracellular flux technology now offer an opportunity to perform these measurements with high specificity and sensitivity. This chapter focuses on the role of the reserve capacity in response to the reactive lipid 4-hydroxynonenal (HNE).

As discussed in Chapter 1, HNE is a reactive aldehyde that is produced endogenously as an end product of lipid peroxidation. The structure of HNE is shown in Figure 4-1 along with the structure of nonanal, a non-electrophilic 9-carbon analog of HNE that is used in this chapter as a control for HNE treatment. The absence of the electrophilic carbon prevents binding to proteins, making nonanal only reactive as an aldehyde.
Figure 4-1: Structure of HNE and Nonanal. Shown above are the structure of the two lipids used in this chapter. HNE has a single electrophilic carbon denoted by the asterisk in the top structure. Nonanal is a non-electrophilic 9-carbon lipid used as a structural control for HNE.
HNE is known to be increased in hearts following ischemia-reperfusion injury [87, 88], and is thought to contribute to the pathology that follows this injury [89, 147, 148]. The experiments presented in this chapter examine the effects of HNE on cellular bioenergetic parameters in intact isolated neonatal rat ventricular myocytes (NRVM). A role for HNE in altering cellular bioenergetics has been proposed by many researchers, however these studies have focused on the properties of mitochondria isolated from hearts treated with exogenous HNE or stimulated to produce increased endogenous HNE [89, 92, 94, 149, 150]. These studies point to the importance of understanding how changes in isolated mitochondria translate to changes in bioenergetic events that take place in the intact myocardium during ischemia-reperfusion and heart failure. Cardiac tissue is rich in mitochondria, which are capable of dynamically responding to energy demand for increased work. This oxidative phenotype allows for rapid and substantial ATP production for cardiac function. It is clear from experiments using $^{31}$P NMR that even under an increased work load in the physiological range, cardiac mitochondria appear to have a substantial “reserve capacity.” These experiments utilized the spin resonance of phosphorus to quantify the levels of ATP, free inorganic phosphate ($P_i$), and phosphocreatine (PCr) [151]. Similar experiments have shown that brief vigorous exercise leads to a decrease in the high energy phosphate (HEP) present as phosphocreatine and ATP, and an increase in free inorganic phosphate [152, 153]. Thus, the cells are using this reserve capacity in response to increased work load. This reserve capacity has been shown to be depleted in patients with defects in oxidative phosphorylation machinery [154], and under conditions of severe stress such as pressure overload or ischemia [155, 156].
Role of the Reserve Capacity in Heart Failure

Studies using mitochondria isolated from animals with experimentally-induced heart failure typically show changes in the activity of the respiratory chain, but the functional impact remains unclear once the organelles are removed from their cellular context [157-160]. It is frequently the case that the activity of respiratory chain complexes of isolated mitochondria such as complex I are decreased in the diseased heart [161, 162]. However, it is challenging to extend these changes seen in mitochondria to the cellular setting since the cytosolic control over metabolism is lost in isolated mitochondria. For example, it is known that the maximal capacity for oxidative phosphorylation is higher than that used under normal conditions [156]. This raises the question of the role of this reserve capacity and leads to the hypothesis that it is required by cells to respond to stress and that pathological events occur when this bioenergetic reserve is depleted.

The requirement of the reserve capacity for the response to stress, such as occurs in the ischemic and failing heart, has not been examined in intact cardiovascular cells or tissues. Since the diseased heart is associated with increased oxidative stress, exposure of cells to reactive species generated during pathology can be used to test the role of oxidative stress in myocyte dysfunction. The significance of these reactive species is evidenced by studies showing that overexpression of antioxidant enzymes such as Mn-superoxide dismutase (SOD) [163], catalase [164], extracellular-SOD [165], or glutathione peroxidase [166] protects the heart from ischemia-reperfusion injury. Moreover, partial deficiency of Mn-SOD [167] or the complete absence of glutathione peroxidase [168] or CuZn-SOD [169] renders the heart more sensitive to injury. This framework of enzy-
matic antioxidant defense systems and their relevance to the mitochondria is shown schematically in Figure 4-2. This figure depicts the above mentioned detoxification pathways, and demonstrates the relevant reactive species. Importantly, mitochondria are both a source and target of the ROS and RNS produced during cardiovascular disease. The production of hydrogen peroxide can also lead to the induction of lipid peroxidation. Lipid peroxides can then lead to the production of HNE from the arachidonic acid present in mitochondrial membranes. Additionally, volume and pressure overload [170-172] as well as ischemia [173, 174] result in an increase in the consumption of oxygen in myocytes. This increased OCR might suggest a mechanism for further depletion of the bioenergetic reserve capacity in an environment where oxygen availability may already be limiting.

**4-Hydroxynonenal in the Failing Heart**

Secondary products of oxidative stress such as 4-hydroxynonenal (HNE) are normally detoxified by energy requiring processes; however, under pathological conditions, these detoxification pathways fail leading to accumulation of oxidized lipids [89] that can damage key proteins in the mitochondrial respiratory chain. In humans and animal models, downstream products of oxidative stress, such as oxidized lipids, are abundant in the ischemic [88, 89] and failing heart [175, 176]. Therefore, the generation of lipid peroxidation products capable of reacting with cellular nucleophiles could be primary instigators of tissue injury [81]. The \(\alpha,\beta\)-unsaturated aldehydes (e.g., HNE) are likely to be the most significant because they modify proteins which affect energy production [92, 93, 177-181] and cell death pathways [182, 183]. This is corroborated by studies showing
Figure 4-2: Reactive species generation and detoxification in the heart. There are several sources of Reactive Oxygen, Nitrogen, and Lipid Species in cardiac tissue that have been proposed to impact on mitochondrial function. Importantly, the mitochondria are recognized as both a source and target of reactive species during the progression of heart failure and ischemia/reperfusion (I/R) injury. To cope with these reactive species, multiple enzymatic antioxidant pathways exist. Studies using transgenic overexpression of MnSOD, EcSOD, Catalase and Glutathione Peroxidase (GPx) all demonstrate protection in I/R models. Similarly, animals lacking GPx or CuZnSOD are more susceptible to these types of injury. Furthermore, the induction of lipid peroxidation can lead to the production of reactive aldehydes such as 4-hydroxynonenal, leading to downstream protein damage.
that activation of a key enzyme required for the mitochondrial detoxification of HNE [89] protects the heart from injury [184, 185]. As mentioned above, studies of isolated mitochondria are often difficult to extend to the intact cellular context when examining the impact of a compound that adducts proteins in both the mitochondrial and cytosolic compartment. Examination of the distribution of HNE in NRVM following treatment with 10 μM HNE demonstrates that there is little localization to a single subcellular compartment (Figure 4-3). This experiment was performed using an anti-protein-HNE adduct antibody with fluorescent secondary detection under confocal microscopy. As shown in Figure 4-3, green fluorescence indicating HNE adducts is diffuse throughout the entire cell. There is some accumulation near the plasma membrane, and this may be due to the lipophilicity of HNE itself. Liu and colleagues reported that HNE can localize to plasma membranes rapidly following exogenous administration of HNE [186], and others have reported the HNE can migrate to the nucleus over time [187]. Thus, HNE is likely affecting proteins in cellular compartments other than the mitochondria, despite reports of adducts present there. Together, these data support the conclusion that HNE could be impacting upon cellular bioenergetics through the modification of proteins outside the mitochondrial compartment, influencing substrate availability or glycolysis.

Two important questions are thus raised by these findings: 1) Does bioenergetic reserve capacity exist in cardiac myocytes and 2) Does it modulate the response to stress associated pathology? The recent availability of the techniques discussed in Chapter 2 allows the non-invasive measurement of mitochondrial respiration and glycolysis now offers the opportunity to address these questions. Therefore, we hypothesized that
Figure 4-3: HNE-protein adducts are not localized to a specific cellular compartment. Neonatal rat ventricular myocytes were left untreated, or treated with 10μM HNE for 1 h. The cells were then fixed and permeabilized before blocking for 1 hour with 10% goat serum. The cells were then probed using an Anti-HNE antibody (Panels A and B), control IgG (Panel C), or no primary antibody (Panel D). Fluorescent secondary antibodies were then used to detect the primary antibodies (1:1000 Chicken anti-rabbit AF488). Shown are representative images for the untreated (A), and HNE treated cells (B), as well as cells treated with HNE, but probed with IgG only instead of anti-HNE antibody (C), and cells probed with the fluorescent secondary for the HNE antibody only (D).
maintenance of mitochondrial function and the availability of a bioenergetic reserve capacity is critical to combat oxidative stress and that, when exceeded, protein damage and cell death occurs. To test this hypothesis, we quantified the bioenergetic changes that occur in intact cardiac myocytes exposed to HNE. By measuring XF, we were able to measure oxygen consumption and proton production, indicative of oxidative phosphorylation and glycolysis, respectively, in intact rat neonatal ventricular myocytes exposed to HNE.

The data presented in this chapter support the hypothesis that pathologically relevant concentrations of oxidized lipids exhaust the reserve capacity of mitochondria. When this capacity is depleted, cellular injury occurs accompanied by decreased mitochondrial oxygen consumption, decreased efficiency due to proton leak, and increased protein-HNE adduct formation. These results directly demonstrate the presence of a bioenergetic reserve capacity in intact myocytes. Furthermore, these findings suggest that oxidized lipids such as HNE, which accumulate in the heart during ischemia and heart failure, could cause, accelerate, or worsen myocardial injury by diminishing this reserve capacity.

**RESULTS**

**Measuring Bioenergetic Function in Intact Neonatal Rat Ventricular Myocytes**

In the first series of experiments, the optimal seeding density of neonatal rat ventricular myocytes (NRVM) was determined. Similarly to the method described in Chapter 3 for Bovine Aortic Endothelial Cells, NRVM were seeded into collagen-coated Sea-
Figure 4-4: Measurement of bioenergetic parameters in myocytes using extracellular flux technology. Oxygen consumption rate (OCR) from isolated neonatal rat ventricular myocytes (NRVM): NRVM were seeded at 25,000–75,000 cells/well and the OCR was measured. Linear regression analysis of the data was performed and is shown here. The r² value for the least squares linear regression is 0.9987. Data shown are the means ± sem. n≥3 per group. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society.
http://www.biochemj.org
horse Bioscience V7 Tissue Culture Plates in growth medium containing 15% fetal bovine serum (FBS) for the first day. After 24 hours, the medium was replaced, and cells were grown in the culture growth medium without FBS. Within 1–2 days of isolation, a confluent monolayer of spontaneously beating NRVM formed, and cells were used to determine the appropriate seeding density for the remainder of the experiments. As shown in Figure 4-4, NRVM seeded to 25,000, 50,000, and 75,000 cells/well exhibited remarkable linearity in the basal OCR. Based on this data, the 75,000 cells/well group was chosen as the optimal density, and was used for the remainder of the studies presented in this chapter.

**Protein HNE Adducts and Cell Death Increase With HNE Treatment**

In the next series of experiments, we measured protein-HNE adduct formation in response to exogenous treatment with this reactive compound. Importantly, many researchers have shown that protein-HNE adducts are increased in isolated mitochondria in response to oxidative stress [91, 179, 181, 188]. In order to maintain concordance, and relevance in light of these studies, we sought to replicate this in intact NRVM. NRVM were seeded at 75,000 cells/well and then treated with either HNE or the non-electrophilic 9-carbon lipid analog of HNE, nonanal, for 2 h (Figure 4-1). For these experiments, we used concentrations of HNE that have been shown to occur in the ischemic and failing heart [89, 175, 189, 190]. The cells were harvested and proteins were separated by SDS-PAGE followed by Western blotting with anti-protein-HNE antibodies. As shown in Figure 4-5 Panels A and B, HNE-protein adducts accumulated in a concentration-dependent manner following this treatment. One proposed mechanism of HNE-
Figure 4-5: HNE promotes protein damage. NRVM were treated with the indicated concentrations of HNE or nonanal for 90 min for measurement of protein-HNE adducts. (A) Western blots of HNE-modified proteins: After treatment with the indicated concentrations of HNE, myocytes were lysed, and protein-HNE adducts were detected by immunoblotting. Actin was used as a normalization control for Western blotting experiments, and nonanal was used as a non-electrophilic analog control for HNE. Representative blots are shown. (B) Relative quantification of protein-HNE modifications: Protein-HNE antibody immunoreactivity was quantified by densitometry, and the fold change of immunoreactivity over non-treated cells was plotted as a function of HNE concentration. Cells receiving 10μM nonanal displayed minimal reactivity (□). Data shown are the means ± sem. n=3 per group. *p<0.01 vs. untreated cells. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. http://www.biochemj.org
**Figure 4-6: HNE causes cell death in NRVM.** (A) Cell death assay of HNE-treated myocytes: NRVM were exposed to 0–30 μM HNE for 8h and photomicrographs were acquired for the indicated groups. Representative images are shown. (B) Measurement of cell death by the MTT assay was performed immediately following image acquisition. Data shown are means ± sem, n≥3; *p<0.01 vs. non-HNE treated myocytes from each time point. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. [http://www.biochemj.org](http://www.biochemj.org)
induced protein adduct formation is through increased oxidative stress secondary to treatment with HNE. To exclude the involvement of this mechanism here, the structural analog of HNE, nonanal was used. Nonanal does not react with proteins, and as shown in Figure 4-5 Panel A, did not promote the formation of protein-HNE adducts. This suggests that the increase in HNE adducts following exogenous administration of HNE is not due to secondary oxidant stress induced by the treatment regimen.

Additionally, cell death as a result of the HNE treatment was examined 8 h post-treatment by the MTT assay. HNE exhibited both a concentration and time dependent effect on NRVM viability (Figure 4-6 A and B). Photomicrographs of NRVM treated with 0-20 μM HNE are shown in Panel A. After 8 h, the assay media was replaced with media containing 0.4 mg/ml MTT reagent as described in Chapter 2. After an additional 2 h, the media was removed, and resulting formazan crystals were solubilized using DMSO. The absorbance was read and cell viability was expressed as the percent of vehicle treated cells. Importantly, this index of cellular survival is dependent on mitochondrial function, and can possibly indicate a decrease in overall mitochondrial function following HNE treatment at these time points.

**HNE Depletes Intracellular Glutathione**

HNE can be metabolized by a number of enzymes in the cell including the glutathione (GSH) dependent S-transferases [191]. Accordingly, we measured total GSH (GSH +GSSG) levels in cell lysates treated with HNE for 90 min. This was performed using the Tietze recycling method for GSH determination [110] and is a useful method
Figure 4-7: Total glutathione content is depleted following HNE treatment. Total GSH content was measured in NRVM cell lysates exposed to 0-20μM HNE for 90 min. Data are expressed as the nmol GSH/mg protein. All data shown are means ± sem, n≥3; *p<0.01 vs. non-HNE treated myocytes from each time point. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society.

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for quantification of total GSH in the cell following HNE treatment. Because HNE is potentially detoxified through GSH conjugation, this technique allows for determination of the total glutathione which has not been conjugated to HNE. It is then of note that utilizing other techniques for this measurement, such as electrochemical detection of reduced and oxidized glutathione might yield falsely inflated values for the effects of HNE. This would be a direct consequence of reporting GSH/GSSG ratios in that HNE would preferentially decrease GSH levels, biasing the data towards a more highly oxidized glutathione result.

Figure 4-7 demonstrates that at HNE concentrations as low as 2.5 μM, total GSH levels are decreased as anticipated. Notably, this effect was maximal at the lowest concentration of HNE tested. This suggests that GSH depletion is not the mechanism of cell death reported in Figure 4-6. Because cell death increases dose dependently with HNE, and shows no appreciable loss of viability at 5 μM or below, it is unlikely that GSH depletion is playing a substantial role in the mechanism. Additional support for this concept is shown by the protein-HNE adduct data. Figure 4-5 demonstrated that the increase in protein-HNE adducts occurs dose dependently with increasing HNE concentration, and it is thus unlikely that the GSH depletion plays a role in regulating this process.

**Effect of HNE on aerobic metabolism in intact neonatal cardiomyocytes**

To determine if bioenergetic derangements preceded cell death due to HNE, we assessed oxygen consumption in NRVM treated under the same conditions as shown in Figure 4-5. After basal oxygen consumption measurements, HNE was injected to give
Figure 4-8: HNE increases oxygen consumption in isolated myocytes. Oxygen consumption rate (OCR) plots from myocytes exposed to 0–30 μM HNE: The basal OCR was measured followed by addition of 0 (filled squares, solid line), 5 (open squares, solid line), 10 (filled squares, dashed line), 20 (open squares, dashed line), or 30 μM HNE (filled squares, dotted line), as indicated by the arrow. Mitochondrial oxygen consumption was then measured for the indicated time. The OCR values are shown as the percent of baseline for each group. For visual clarity, statistical indicators were omitted from the graph. These data are analyzed in more detail in Figure 4-7 where statistical significance is indicated. Data shown are means ± sem, n≥3. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. http://www.biochemj.org
final concentrations of 0–30 μM. Control wells received vehicle (ethanol) or the structural control for HNE, nonanal (10 μM), in DMEM. As shown in Figure 4-8, HNE initially increased OCR in a concentration dependent manner. Myocytes treated with the lowest concentration of HNE tested (5 μM) demonstrated a steady increase in the rate of mitochondrial oxygen consumption which plateaued after approximately 150 min of HNE exposure. Exposure to higher concentrations of HNE (10–30 μM) resulted in a maximal OCR that declined thereafter. Apparent maximal increases in OCR were reached with 10, 20, and 30 μM HNE approximately 130, 70, and 35 min after injection, respectively.

In order to more clearly understand the dependence for HNE to stimulate the consumption of oxygen, the rate of stimulation of OCR was next plotted as a function of HNE treatment concentration. The traces from the OCR measurements shown in Figure 4-8 were analyzed to determine the rate of OCR stimulation from the time of HNE injection to the peak OCR measurement for each concentration independently. Interestingly, the rate of increase in OCR increased linearly with HNE concentration, and is shown in Figure 4-9 Panel A. Additionally, to quantify the overall increase in the amount of oxygen consumed by the myocytes due to each treatment, the OCR area under the curve for each group was calculated by multiplying the group average by the time interval for that rate and subtracting the baseline oxygen consumption rate. The resulting value can be expressed in pmoles of oxygen consumed during the assay after injection of vehicle, nonanal, or HNE. As shown in Figure 4-9 Panel B, exposure of the myocytes to HNE at 5–10 μM concentrations resulted in a stimulation of approximately 20 nmoles of oxygen
Figure 4-9: HNE increases oxygen consumption in isolated myocytes. Oxygen consumption analysis plots from myocytes exposed to 0–30 μM HNE: (A) The slope of the initial increase in OCR due to HNE treatment was plotted as the rate of OCR increase; y=0.1065x + 0.1283, r²=0.99. (B) Area under the curve analyses were used to determine the overall amount of oxygen consumed with each treatment as shown in Figure 4-6. Inset: the shaded area represents the oxygen consumption used to calculate the area under the curve. Data shown are means ± sem, n≥3. *p<0.05 vs. cells not treated with HNE; #p<0.05 vs. cells treated with 5–10 μM HNE. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society.

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consumed during the course of the experiment. This consumption is decreased in the 20-30 μM groups due to the transient increase in OCR that declines over time, falling well below baseline (Figure 4-8). The inset depicts the method of calculation used to determine the area under the curve. Control and 10 μM HNE groups are shown for clarity. The shaded region indicates the area determined as the stimulation of oxygen consumption by HNE, and is representative of the data shown in Figure 4-9 Panel B.

Because the endogenous production of HNE due to lipid peroxidation could be increased through the induction of a general cellular stress response, the next series of experiments examined the effects of the treatment scheme on OCR induction. To examine this, NRVM seeded as in the above experiments were treated with the non-reactive structural analogue nonanal. Nonanal is a 9-carbon lipid that lacks the functional ability to adduct proteins. As shown in Figure 4-10, 10 μM nonanal injection had no effect on OCR, whereas 10 μM HNE stimulated OCR as seen in the above experiments. This observation fits well with the inability of nonanal to increase protein-HNE adducts (Figure 4-5).

One possible mechanism to explain the induction of OCR by HNE is that contraction rate or force is stimulated upon treatment. NRVM are known to spontaneously contract when in culture, and contraction in myocytes is reported to account for most of the oxygen consumed by these cells [192-194]. In the next series of experiments, we utilized a well-known inhibitor of myocyte contraction, 2,3-butanedione-2-monoxime (BDM), to address this possible route of OCR stimulation [195, 196]. As shown in Figure 4-11, in-
Figure 4-10: The structural analogue of HNE, Nonanal does not increase OCR. HNE or Nonanal (10 μM) was injected following three basal measurements of OCR in NRVM. OCR was measured for approximately 2 h thereafter. The OCR values are shown as the percent of baseline for each group. For visual clarity, statistical indicators were omitted from the graph. Data shown are means ± sem, n≥3.
Figure 4-11: BDM partially prevents HNE-stimulated oxygen consumption in isolated myocytes. The basal OCR was measured followed by addition of vehicle control (EtOH, filled squares, solid line), 10 μM HNE (filled squares, dashed line), 10 mM BDM (filled circles, dotted line), or 10μM HNE + 10 mM BDM (open circles, dotted line). Additions were given where indicated by the arrows. OCR values are shown as the percent of baseline for each group. For visual clarity, statistical indicators were omitted from the graph. Data shown are means ± sem, n≥3.
Figure 4-12: Inhibition of mitochondrial calcium uptake by ruthenium red has no effect on HNE-stimulated oxygen consumption. The basal OCR was measured followed by addition of vehicle control (EtOH, filled squares, solid line), 10 μM HNE (filled squares, dashed line), 10 μM ruthenium red (filled circles, dotted line), or 10μM HNE + 10 μM ruthenium red (open circles, dotted line). Additions were given where indicated by the arrows. OCR values are shown as the percent of baseline for each group. For visual clarity, statistical indicators were omitted from the graph. Data shown are means ± sem, n≥3.
jection of BDM approximately 1 h after HNE partially inhibited the stimulation of OCR by HNE. Importantly, BDM inhibited a large amount of the basal OCR in cells not treated with HNE. This indicates that there is some basal OCR that is used for cell contraction; however, increased contractility or contraction rate is not fully responsible for the stimulation of OCR by HNE.

An alternative explanation for the increase in OCR could be an increase in Ca\(^{2+}\) influx into the mitochondria. Increased Ca\(^{2+}\) import into the mitochondrial matrix has been reported to increase oxygen consumption and ATP production in myocytes, and this was inhibitable by Ca\(^{2+}\) transport blockers [197-199]. To determine if the increase in OCR is dependent on Ca\(^{2+}\) influx into the mitochondria, NRVM treated with HNE were injected with 20 \(\mu\)M ruthenium red (RuRed) approximately 1 h after HNE in a manner similar to the above experiment using BDM. This concentration of RuRed has been reported to inhibit Ca\(^{2+}\) influx into cardiac myocytes by blocking the mitochondrial Ca\(^{2+}\) uniporter [200]. As shown in Figure 4-12, RuRed had no effect on the OCR stimulated by HNE. In addition, RuRed had no effect on the basal OCR in these cells, likely indicating that basal Ca\(^{2+}\) transients play little role in controlling mitochondrial function in these intact NRVM.

**Effects of HNE on glycolysis**

In addition to OCR, the XF assay also allows the measurement of protons that were produced by the cells, which reflects lactate production and is therefore an index of glycolysis [60, 201]. Figure 4-13 shows the extracellular acidification rate (ECAR) pro-
Figure 4-13: HNE increases glycolytic rate in isolated myocytes. Measurements of proton production from myocytes exposed to 0–30 μM HNE: The basal extracellular acidification rate (ECAR) was measured three times, and then followed by addition of 0 (filled squares, solid line), 5 (open squares, solid line), 10 (filled squares, dashed line), or 20 μM HNE (open squares, dashed line), as indicated by the arrow. The rates of extracellular acidification, indicative of changes in glycolytic flux, were then measured for the indicated time. For visual clarity, the 30 μM HNE group and statistical indicators were omitted from this graph. These data are analyzed in more detail in Figure 4-10 where statistical significance is indicated. Data shown are means ± sem, n≥3. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. [http://www.biochemj.org](http://www.biochemj.org)
Figure 4-14: HNE increases glycolytic rate in isolated myocytes. Measurements of proton production from myocytes exposed to 0–30 μM HNE: (A) The slope of the initial increase in ECAR due to HNE treatment was then measured and plotted as the rate of ECAR increase; \( y=0.0534x + 0.2751 \), \( r^2=0.97 \). (B) Area under the curve analyses of the proton production rate were used to determine the overall amount of protons produced with each treatment. Data shown are means ± sem, \( n \geq 3 \). * \( p < 0.05 \) vs. cells not treated with HNE; # \( p < 0.05 \) vs. cells treated with 7.5 and 10 μM HNE. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. http://www.biochemj.org
file of control myocytes and myocytes treated with 0–30 μM HNE. Similar to OCR, HNE increased the ECAR in a concentration and time-dependent manner. Analogous to the OCR results (Figure 4-8), ECAR showed a biphasic response in the 10 and 20 μM HNE groups, indicating severe damage to bioenergetic components at later time points. Additional analysis regarding the degree of stimulation of ECAR by HNE was performed as in the OCR analysis. As shown in Figure 4-14 Panel A, the initial rate of increase in ECAR was linear and dependent on HNE concentration. Interestingly, the time to maximal stimulation of ECAR was notably longer than the OCR measurement. This indicates that the stimulation of these pathways is occurring by one of two mechanisms. The first possibility is that the stimulation of OCR and ECAR are occurring through independent processes involving modifications of different proteins that have different kinetic parameters. A second possibility is that the response in the ECAR rate is a direct consequence of the stimulation of OCR. Possible support for this second concept will be discussed in further detail below. Similar to the data shown for OCR in Figure 4-9 (Panel B and inset), the area under the curve of the proton production rate (PPR) was calculated to determine the overall increase in proton production over the time course of the experiment. HNE treatment resulted in an increase in proton production, indicative of an increase in glycolysis, over the course of the experiment (Figure 4-14 Panel B). The maximal rate of proton production occurred in cells treated with 7.5–10 μM HNE.

Identification of mitochondrial defects incurred by HNE

To determine the specific mitochondrial derangements that occur in response to HNE, we next used the mitochondrial function assay described in Chapter 2. For these
Figure 4-15: HNE stimulates oxygen consumption, but also causes impairments in OCR over time. After measurement of the basal OCR, HNE was injected to 0 (filled squares, solid line), 5 (open squares, solid line), 10 (filled squares, dashed line), or 20 μM (open squares, dashed line) final concentrations. The mitochondrial function assay was then performed by sequential injections of oligomycin, FCCP, and antimycin A to determine the level of proton leak and ATP-linked oxygen consumption, the maximal OCR, and the non-mitochondrial OCR, respectively. Data shown are the means ± sem, n≥3. Statistical indicators are omitted here for visual clarity. These data are analyzed in more detail in Figures 4-12, and 4-13 where statistical significance is indicated. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. [http://www.biochemj.org](http://www.biochemj.org)
experiments, NRVM treated with HNE to final concentrations of 0-20 μM were exposed to oligomycin, FCCP, and antimycin A at the time points indicated (Figure 4-15). The measurements taken after each injection were used to calculate the ATP-linked OCR, proton leak, reserve capacity, and non-mitochondrial OCR at 2h after the addition of HNE. As shown in Figure 4-15, OCR was stimulated in response to HNE consistent with the data presented earlier in this chapter. Additionally, this experiment demonstrates that the control cells are able to respond to the oligomycin, FCCP, and antimycin A sequential injections as predicted, and as described in Chapter 2.

To examine the specific mitochondrial parameters affected by HNE, the contribution of ATP synthesis, proton leak, and non-mitochondrial oxygen consumption were determined as a fraction of the basal rate. Also, the reserve capacity was calculated from the FCCP-stimulated rate. Figure 4-16 shows the result of these calculations. The stimulation of OCR by HNE was first plotted as the increase in OCR from baseline of each individual group, measured at the last date point prior to injection of oligomycin. This data is shown as a function of HNE concentration in Figure 4-16 Panel A. Upon addition of oligomycin, Figure 4-16 Panel B shows that the OCR in the presence of HNE increased significantly by up to 3-fold at the 10 μM concentration. Proton leak was increased as by HNE in a concentration-dependent manner (Figure 4-16 Panel C). Interestingly, the rate of ATP-linked oxygen consumption showed a biphasic response (Figure 4-16 Panel D), where it increased by approximately 3-fold with 10 μM HNE and decreased at 20 μM. These results suggest that HNE increases OCR both by increasing proton leak (thereby decreasing mitochondrial efficiency) and by increasing energy demand.
Figure 4.16: Specific defects in mitochondrial function caused by HNE. The HNE-induced changes in the following parameters derived from the data in Figure 4.9 are shown: (A) The OCR induced by HNE (B) The Oligomycin-insensitive OCR (C) The OCR ascribed to proton leak (D) The OCR ascribed to ATP-synthesis (E) bioenergetic reserve capacity, and (F) the non-mitochondrial OCR. Data represent means ± sem. n ≥ 3/group. *p<0.05 vs. myocytes not treated with HNE. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society. http://www.biochemj.org
Next, the maximal respiratory rate was determined from the FCCP-stimulated rate. As shown in Figure 4-15, HNE at 0–10 μM concentrations had no effect on this maximal respiratory rate. However, concentrations of HNE in excess of 10 μM resulted in inhibition of oxygen consumption even after FCCP addition, suggesting overt damage to mitochondrial respiratory complexes (Figure 4-15). The reserve capacity was calculated based on the OCR immediately prior to oligomycin addition and the maximal respiratory rate. As shown in Figure 4-16 Panel E, HNE decreased the mitochondrial reserve capacity in a concentration-dependent manner consistent with damage to the respiratory chain associated with increased protein adducts (Figure 4-5 Panel A). The rate of oxygen consumption due to non-mitochondrial sources was determined in the presence of HNE using antimycin A and was unchanged (Figure 4-16 Panel F).

**HNE Stimulates Energy Production: The Metabolic Image**

Approximately 70 min after HNE addition (indicated by the dotted line in Figure 4-15), the ECAR and OCR were used to obtain a “metabolic image” of the cells in each treatment group. The image is divided in four quadrants, which relate the relative activities of the glycolytic and aerobic metabolism in response to HNE. As shown in Figure 4-17, HNE concentration-dependently stimulates both glycolysis and the aerobic consumption of oxygen, consistent with an increased energy demand of the cells as they respond to this stress.
Figure 4-17: Changes in the mitochondrial and glycolytic profiles due to HNE treatment. Metabolic profile of the stimulatory effect of HNE on aerobic and anaerobic respiration: The OCR and ECAR were plotted against one another at the time where OCR was increased to the greatest extent in cells treated with 20 μM HNE (from experiment in Figure 4-11 – dotted line at ~70 min timepoint). Data shown are the means ± sem. *p<0.05 vs. cells not treated with HNE; #p<0.05 vs. cells treated with 5 μM HNE; @p<0.05 vs. cells treated with 10 μM HNE. Reproduced with permission from Hill BG, Dranka BP, Zou L, Chatham JC, and Darley-Usmar VM., 2009, Biochem. J., 424(1), 99-107, © the Biochemical Society.

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Respiratory State\textsubscript{apparent} is Decreased by HNE

To examine the total effect of the mitochondrial derangements examined in the above figures, the State\textsubscript{apparent} was calculated for cells treatment with HNE. Because the inhibitory phase of HNE on cellular oxygen consumption happens rapidly in response to high doses of HNE, we sought to quantify this respiratory parameter during the early, adaptive phase of the response to HNE. In this experiment, NRVM were treated as described above with HNE (0-30 μM) for just 30 min. In response to this treatment, OCR and ECAR began to increase immediately and dose-dependently in concordance with the experiments shown in Figure 4-8 and 4-13. The cells were then sequentially treated with oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) as described in Chapter 2. From these measurements, the State\textsubscript{apparent} was calculated using the final HNE-stimulated OCR rate prior to oligomycin injection, and the FCCP-stimulated rates as the “basal” and “FCCP” rates in Equation 2-1. The results of this calculation are shown in Figure 4-18. HNE dose-dependently decreases the State\textsubscript{apparent}, which approaches a State 3-like condition with increasing HNE concentration. This, taken with the data indicating that mitochondrial efficiency is decreased, likely shows that the mitochondria are working harder in the presence of HNE than in the basal condition. The implications of this finding will be discussed in further detail below.

DISCUSSION

This chapter utilized extracellular flux analyses to evaluate the effects of the electrophilic lipid HNE on bioenergetic function in intact myocytes. Importantly, the data discussed in this chapter differ from that shown in Chapter 3 in that an excitable cell type
Figure 4-18: Respiratory $\text{State}_{\text{apparent}}$ decreases with increasing HNE concentration. NRVM examined in an experiment similar to that shown in Figure 4-11 were used for determination of $\text{State}_{\text{apparent}}$. Cells were treated with the indicated concentration of HNE for 30 min. and OCR was measured. The final HNE-stimulated OCR rate prior to oligomycin administration and the FCCP-stimulated OCR were used for this calculation. Data shown are the means ± sem. n≥3 per group. *p<0.05 vs. cells not treated with HNE.
was used. This allowed for novel insight into the mechanisms by which cells consume oxygen in response to stressors, and more importantly, gave an indication of the importance of the reserve capacity in these cells. This data demonstrates for the first time that oxidized lipids, at concentrations that accumulate under pathophysiological conditions in the heart, increase cardiomyocyte oxygen consumption and deplete the bioenergetic reserve capacity. We found that HNE stimulated the rate of cellular oxygen consumption in a concentration-dependent manner. This increase in oxygen consumption was due to both increased bioenergetic demand placed on the cardiac myocytes and decreased mitochondrial efficiency (i.e., proton leak). Glycolytic flux was also increased in response to HNE treatment, underscoring the integrated nature of the metabolic response to energy demand and expenditure in the presence of reactive lipid species. The HNE-induced increase in oxygen consumption was not compensated by an increase in the maximal respiratory capacity of the myocytes; consequently, HNE treatment resulted in depletion of the bioenergetic reserve capacity, overt respiratory failure, and cell death.

Reliance on Oxygen Consumption in the Heart

In the heart, mitochondria make up at least 20% of the myocyte volume [202, 203] and provide the unremitting energy required for contraction. Aerobic respiration is therefore indispensable to sustain cardiac function and viability. At rest, the heart consumes up to 0.15 ml O₂/min/g tissue, which increases several fold with vigorous exercise by calling upon the reserve capacity of the mitochondria and glycolysis [204-206]. Interestingly, the diseased heart requires more oxygen to meet energy needs, which has led to the hypothesis that a state of energy starvation may underlie myocardial pathology [207].
In support of this concept, myocardial oxygen uptake is increased 2-fold in patients with left ventricular hypertrophy over normal subjects [170]. Additionally, researchers have demonstrated an increase in the myocardial uptake of oxygen in experimental hypertrophy, congestive heart failure, and diabetes [171, 172, 208]. Furthermore, the postischemic heart uses more oxygen than the preischemic heart [174], and myocytes subjected to hypoxia-reoxygenation have an increased demand for oxygen [173]. It is reasonable to conclude, therefore, that bioenergetic dysfunction and the imbalance of oxygen supply versus demand is a basic defect in myocardial pathologies. However, the mechanisms underlying this increased demand for oxygen in the diseased heart are unknown. Increased tissue levels of reactive species such as HNE are associated with both acute and chronic cardiac disease [88, 89, 189, 190]. If, as shown here, such reactive species increase the rate of oxygen consumption and decrease bioenergetic reserve capacity in the diseased heart, this could account for the contractile dysfunction associated with pathology.

**HNE as a Secondary Mediator of Oxidative Stress in Cardiovascular Disease**

While the idea that ROS play a fundamental role in cardiovascular disease is well accepted, the molecular mechanisms by which oxidative stress cause cardiac injury are not well defined. ROS can damage cellular components by direct oxidation; however, given the susceptibility of polyunsaturated fatty acids to oxidative attack, there is a strong rationale for invoking the involvement of secondary products of lipid oxidation such as HNE and acrolein in ROS-related injury. Indeed, adducts of such oxidized lipids with proteins have been detected in the diseased heart [88, 89, 92, 180, 190, 209-211] and my-
ocytes exposed to similar compounds capable of modifying proteins promote a phenotype akin to myocardial stunning [212]. It remains unclear, however, whether oxidized lipids or their protein adducts are footprints of unquenched free radicals or if they indeed cause derangements in signaling or bioenergetics.

Recent studies showing that activation of enzymes critical for HNE detoxification protects the heart from ischemic injury suggest that electrophiles are significant contributors to myocardial damage [163, 184, 185]. Many studies have shown that HNE damages electron transport chain complexes and other mitochondrial proteins [91, 95, 157, 177-181], affecting both respiration and critical events such as calcium-induced permeability transition [183, 213]. These studies, however, were limited by their usage of isolated mitochondrial preparations. While useful for understanding putative defects caused by HNE, experiments with isolated mitochondria are generally performed under saturating substrate conditions and are outside their normal intracellular environment. This removes all influence of cell signaling, ion fluxes, and changes in intracellular substrate metabolism (e.g., glycolysis) on bioenergetic regulation. In contrast to the HNE-induced increase in oxygen consumption found here, most studies to date report that HNE primarily inhibits mitochondrial respiration. This is likely due to the fact that the majority of studies have assessed HNE-induced changes in respiration using isolated mitochondrial preparations [89, 177-179].
Effects of HNE in Intact Cardiomyocytes Differ From Studies in Isolated Mitochondria

Here, we demonstrate that HNE increases oxygen consumption and bioenergetic demand and decreases mitochondrial efficiency (Figures 4-8, 4-9, and 4-16). These findings suggest that oxidized lipids contribute to myocardial injury by promoting an energy crisis. In support of this concept, we show that HNE at lower concentrations initially increased the OCR (Figure 4-8), which was due to both an increased proton leak and bioenergetic demand (Figure 4-16). Using the process described in Chapter 2, we can assess the impact of HNE on key bioenergetic parameters in the cell. We have assumed for the purposes of this calculation that the oligomycin insensitive OCR is attributable to proton leak in the basal condition. However, this is not strictly correct since oligomycin has been shown to increase mitochondrial membrane potential and the resulting OCR is likely to be an upper estimate of the contribution from proton leak. While the oligomycin-dependent hyperpolarization has no impact on the conclusion that HNE has increased proton leak (Figure 4-16 Panel C), it will affect the value of the OCR which we attribute to ATP synthesis primarily through an underestimation of this parameter. Nevertheless, it is clear that a significant portion of the stimulation of the basal OCR was due to increased ATP synthesis. The increased proton leak across the inner mitochondrial membrane may be mediated by uncoupling proteins or leakage through damaged respiratory complexes. In support of this, studies using isolated mitochondria have shown that HNE promotes uncoupling and mitochondrial proton leak [93, 94].

The biphasic nature of the HNE-induced changes in oxygen consumption (Figure 4-8) suggests at least two factors contribute to the effects of HNE. Initially, HNE in-
creased the oxygen consumption rate in a concentration-dependent manner (Figure 4-8, 4-9 Panel A). We propose that this is an initial adaptive response to cellular stress induced by HNE and does not involve damage to the respiratory chain. The second phase, where the OCR decreased at the higher concentrations of HNE is likely due to damage to respiratory complexes. This likely occurs due to increased protein adduct formation as shown in Figure 4-5. Another possibility is that endogenous substrates became limiting as OCR increased. However, this is unlikely since 20 and 30 μM HNE, which quickly reached a peak respiratory capacity in the cells (Figure 4-8), resulted in a lower overall consumption of oxygen compared with myocytes treated with 5 and 10 μM HNE (Figure 4-9 Panel B) and decreased the ability of the cells to respire maximally when treated with the uncoupler, FCCP (Figure 4-15). Furthermore, the concentrations of HNE that caused a transient breach of the reserve capacity (i.e., 10–30 μM HNE) were associated with substantially increased protein-HNE adducts, and this resulted in complete cell death (Figure 4-4). As anticipated, GSH was rapidly depleted by HNE and was maximal at the lowest concentration of HNE tested. Since the major bioenergetic changes induced by HNE occurred without a significant change in these repressed GSH levels it is unlikely that GSH depletion plays a major role in the HNE-dependent bioenergetic dysfunction reported here. Together, these data indicate that extensive formation of protein-HNE adducts likely caused damage to respiratory complexes that resulted in inhibition of electron transport.

As discussed above, HNE levels are substantially increased in multiple myocardial pathologies [88, 89, 189, 190, 211], suggesting that HNE promotes tissue damage and
dysfunction by decreasing the bioenergetic reserve capacity. Interestingly, myocardial oxygen consumption in the pressure-overloaded heart is increased to the maximal dinitrophenol-stimulated rate during high workloads produced by catecholamine infusion [156], suggesting that reserve capacity is depleted in the diseased heart under conditions of stress. This is of particular interest since even low dose catecholamine treatment promotes lipid peroxidation and the formation of protein-HNE adducts [211, 214]. While of particular relevance to cardiovascular disease, reduced bioenergetic reserve capacity has also been recognized in neurodegeneration. Specifically, reduction of the spare respiratory capacity has been shown to regulate glutamate excitotoxicity in neurons [215, 216]. Those studies suggested that dysfunction occurs when the ATP demand exceeds the maximal ATP supply put forth by glycolysis and oxidative phosphorylation, thereby implicating that the spare respiratory capacity is critical for maintaining ATP generation under conditions of increased demand [101]. Similarly, we show that the ability of cardiomyocyte mitochondria to respire collapses when the maximal reserve capacity is depleted due to the increase in oxygen consumption by HNE.

Summary

Taken as a whole, these data support the view that products of lipid peroxidation, in the concentration range reported under pathological conditions, contribute to myocardial injury by promoting bioenergetic stress. By evaluating mitochondrial function in intact cells, our findings reveal for the first time a dynamic response of cardiomyocytes to HNE, where oxygen consumption is increased through increased energy demand as well as via non-ATP-linked oxygen sinks (i.e., proton leak). Furthermore, these studies pro-
vide insight into a fundamental mechanism critical to the evolution of myocyte injury, namely the oxidized lipid-induced increase in oxygen consumption and depletion of the bioenergetic reserve capacity. Consequently, these findings have implications not only for our understanding of the pathophysiological processes underlying cardiac disease, but also in other disease states associated with increased oxidative stress.
CHAPTER 5

CHRONIC EXPOSURE TO NITRIC OXIDE DECREASES MITOCHONDRIAL RESERVE CAPACITY IN ENDOTHELIAL CELLS AND INCREASES THE SENSITIVITY TO OXIDATIVE STRESS

INTRODUCTION

The data presented in Chapters 3 and 4 have constituted a view of some of the acute challenges the cardiovascular system can be exposed to during the onset of pathologies such as atherosclerosis or ischemia/reperfusion injury. These chapters have demonstrated a view of the impact of nitric oxide (NO') and 4-hydroxynonenal (HNE) on global cellular bioenergetic status, though the responses examined thus far are only a view of the immediate response to these stressors. In this chapter, the response of endothelial cells to chronic administration of NO' will be more thoroughly examined. NO' is often thought to be of great benefit in the vasculature [77, 78, 114, 217, 218]. Indeed, there have been several studies to examine the role of NO' in vascular protection, and substantial benefit to patients has been reported [219, 220]. The primary mechanisms proposed for this protection is through inhibition of platelet aggregation and vasorelaxation, which increases blood flow to damaged areas. In contrast, the pathogenesis of atherosclerosis is known to be accompanied by an increase in inflammatory cells which produce a number of pro-inflammatory mediators. These cells arrive to an atherosclerotic lesion as macrophages, but quickly engulf oxidized lipids present in the plaque, and take on an appearance that has led them to be called foam cells. These cells are responsible
for much of the inflammatory nature of the progression of atherosclerosis. One protein that is upregulated in these cells is inducible nitric oxide synthase (iNOS or NOS2). Interestingly, iNOS is the only NOS isoform that is not Ca\(^{2+}\)-dependent. This is due to the tight binding of calmodulin to iNOS [221]. The other two NOS isoforms (endothelial NOS and neuronal NOS) both require the binding of Ca\(^{2+}\) to the enzyme, and subsequently have a low normal activity. The lack of a requirement for Ca\(^{2+}\) allows iNOS to produce NO\(^{-}\) in the micromolar level in the vasculature, much higher than typically generated form eNOS or nNOS. Thus the exposure to NO\(^{-}\) in disease states may be much higher than that described in the cytoprotective studies mentioned above. This chapter examines the consequences of chronic, elevated exposure to NO\(^{-}\) in endothelial cells as a model for vascular dysfunction in the progression of cardiovascular disease.

**Nitric Oxide in the Vasculature**

As mentioned above, it is now known that NO\(^{-}\) generated in the vasculature plays important roles in the maintenance of vessel tone and platelet adhesion, as well as other intracellular functions [222, 223]. It is becoming increasingly evident that nitric oxide’s role in cell signaling as both a regulator of apoptosis and as a potential activator of death and survival pathways within the cell is under constant regulation. However, the precise mechanism of NO\(^{-}\) signaling that facilitates apoptosis remains to be determined. The cytoprotective roles of NO\(^{-}\) at low concentrations, or acute conditions have been well documented, and are largely regarded as the domain responsible for the physiological roles that NO\(^{-}\) plays [76, 224]. At extended durations or high concentrations however, NO\(^{-}\) plays a very different role, and has been shown to induce apoptosis in Jurkat leukemic
cells [225, 226] especially in combination with other Reactive Oxygen and Nitrogen Species (ROS/RNS) [227]. Loss of control of this signaling pathway has been shown to have many pathological implications, such as hepatotoxicity [228]. Additionally, NO’ may regulate intracellular levels of ROS/RNS produced by the mitochondria, predominantly through binding to cytochrome c oxidase. Cellular levels of ROS/RNS formation could be a major factor in cell survival in response to oxidative/nitrosative stress [29]. The changes in cell signaling that accompany the response to NO’ treatment are well-defined in our model and will be discussed below. In this chapter, we seek to expand our understanding of the cellular response to NO’ from a bioenergetic view, as well as other functional pathways as they impact on a cell type relevant to vascular function and homeostasis.

The Role of Vascular Endothelial Cell Death in Atherosclerosis

The role of apoptosis in the pathogenesis of atherosclerosis is one of the most interesting areas of research in this disease field. In recent years, the understanding of how endothelial dysfunction relates to the formation and stability of atherosclerotic plaques has been greatly advanced, and is now regarded to be one of the most important initiating points in this disease [229-231]. Several studies have shown that increased apoptosis of the endothelium is inversely correlated to restenosis [232] and plaque rupture [233, 234]. As shown in Chapter 3, acute exposure to NO’ significantly sensitizes endothelial cells to apoptosis when in combination with ROS produced by the redox cycling compound DMNQ. As described previously, endothelial cells are chronically exposed to high levels of NO’ during the pathogenesis of inflammatory diseases such as atherosclerosis. Thus,
understanding the bioenergetic impact of known nitric oxide interactions with the mito-
chondria may be of critical importance in defining the pathological mechanisms that ac-
company atherosclerosis. This theory is heightened given the well-defined role of the 
mitochondrion in the apoptotic cascade.

RESULTS

Chronic Nitric Oxide Decreases Respiratory Enzyme Subunit Protein Levels

In the first series of experiments, bovine aortic endothelial cells (BAEC) were ex-
posed to the NO donor Deta NONOate (Deta NO) for 16 hours at concentrations ranging 
from 0.1 to 2 mM. The cells were then lysed and harvested, and equal amounts of protein 
were then loaded onto SDS-PAGE gels for analysis. Levels of mitochondrial proteins 
were first examined. The cells were first probed for Cytochrome c Oxidase (COX) Sub-
unit I. This is a mitochondrially-encoded subunit of this enzyme, is the largest subunit in 
the complex, contains the reactive centers of the enzyme, and has been shown to correlate 
well with the overall levels of the intact enzyme [63, 76, 235]. As shown in Figure 5-1 
Panel A, levels of this protein are significantly decreased by treatment with the NO do-
nor. In addition, the levels of COX Vb, a nuclear-encoded subunit were also examined. 
COX Vb is of interest as it is implicated in maintaining the structure of the dimeric com-
plex, and is thought to be partially responsible for enzyme regulation and hypoxic sens-
ing. Additionally, this subunit is reported to be crucial for maintaining the structure of 
the enzyme [236]. Data shown in Chapter 6, as well as reports from other researchers 
indicate that the loss of this subunit in human cells leads to decreases in the total enzyme 
levels [237] further indicating that this protein is responsible for holoenzyme structure.
Figure 5-1: Chronic nitric oxide decreases levels of the mitochondrial respiratory chain complex cytochrome c oxidase. Confluent Bovine aortic endothelial cells were treated with increasing concentrations of Deta NONOate for 16 hours. The cells were harvested and probed for Cytochrome c Oxidase Subunits I and Vb. Representative blots are shown in A. Panel B shows quantification of Cytochrome c Oxidase Subunits I (■) and Vb (○) expressed as a fold change compared to the untreated control. Data shown are the means ± s.d. n=3.
Additionally, this figure shows that COX IV levels are decreased in response to NO’.

These Western blots were quantified, and Figure 5-1 Panel B demonstrates the fold decrease in the protein levels as a function of the control treated cells.

**Chronic NO’ Decreases Mitochondrial Membrane Potential**

As one functional consequence of a decrease in mitochondrial proteins, we next examined the effects of chronic exposure to Deta NONOate on the mitochondrial membrane potential. As shown in Figure 5-2, mitochondrial membrane potential is significantly decreased as measured by JC-1 fluorescence. In this experiment, BAEC were grown on chambered coverslips and treated with 500 μM Deta NONOate for 16 hours. The cells were then treated with 7.4 μM JC-1. JC-1 is a planar compound that normally exhibits green fluorescence when excited at 488 nm. When in high concentrations, however, individual molecules aggregate and the fluorescence spectrum exhibits a red shift. Notably, this compound has a depolarized cationic charge, allowing it to accumulate in the mitochondrial matrix according to the Nernst potential. Because a high membrane potential will drive the accumulation of the dye concentration in the mitochondrial matrix, the shift from green to red fluorescence that is seen in control cells can be used to quantify the degree of membrane polarization. Figure 5-2 Panel A demonstrates strong red fluorescence in the control cells, indicating a high mitochondrial membrane potential. Upon treatment with NO’, the red fluorescence is largely absent, and this is quantified in Panel B. Hoechst 33258 was used as a nuclear dye to visualize the nucleus in these live cells. NO’ is known to decrease proliferation of BAEC at the concentrations used here, and thus the data shown are normalized to cell number measured by counting nuclei.
**Figure 5-2: Chronic nitric oxide treatment decreases mitochondrial membrane potential.** Confluent BAEC were treated with 500μM Deta NONOate for 16 hours. The cells were then loaded with JC-1 (7.4μM), and Hoescht 33258 for 15 min. Representative epifluorescence images are shown in panel A. The images were analyzed using Simple PCI software, and the red fluorescence intensity is plotted in panel B. Data shown are the mean ± s.d. n=3. *, p<0.05 vs. CTL.
Chronic NO’ Decreases Cellular Bioenergetic Capacity

In the next series of experiments, the effect of chronic NO’ treatment on cellular bioenergetics was assessed using the methods described in Chapter 2. For these experiments, a slightly different seeding protocol was used. Because these cells are to be treated with NO’ for at least 16 hours, BAEC were seeded to 20,000 cells/well in Seahorse Bioscience V7 tissue culture plates, and then allowed to adhere and grow overnight. The cells were then treated with Deta NONOate (100-1000 μM) in the plates, and allowed to incubate 16 hours in a standard tissue culture incubator. The media was then removed and cells were washed with Assay Medium as described in Chapter 2. The cells were then allowed to incubate in the 37°C, non-CO2 incubator for 1 h until the start of the mitochondrial function assay. Three basal measurements of OCR and ECAR were performed and this was followed by sequential injections of oligomycin (final concentration 1μg/ml), FCCP (final concentration 1 μM), and antimycin A (final concentration 10 μM). A measurement of OCR and ECAR was made following each injection. The data from this experiment are shown in Figure 5-3, Panel A. As shown in this figure, NO’ causes a strong inhibition of oxygen consumption at all concentrations tested, and this effect persisted across the entire experiment. Further analysis of individual mitochondrial function parameters is discussed below.

Importantly, the data shown here are in the absence of NO’ since it was washed out in the change to assay media. This indicates that the inhibition of basal oxygen consumption that is seen in this experiment is not due to a direct binding of NO’ at Cytochrome c Oxidase. Also, the inhibitory effect of NO’ on the FCCP-stimulated OCR per-
Figure 5-3: Chronic NO⁺ treatment decreases cellular oxygen consumption in an NO⁺-independent manner. BAEC were seeded to 20,000 cells per well in Seahorse Biosciences V7 Tissue Culture Plates and allowed to grow for 24 hours. The cells were then treated with the indicated concentration of Deta NONOate for 16 hours. Following treatment, the cells were washed and switched to assay medium for 1 hour, and then the mitochondrial function assay was run as described in Chapter 2. Data shown are the means ± sem. n≥3.
sists, even when the data are recalculated as a percentage of the baseline rate for each treatment group (Figure 5-3 Panel B). This is an important distinction from the data presented with acute NO’ exposure in Chapter 3, and will be discussed further below.

The ECAR of these cells was also measured following NO’ treatment as a surrogate for glycolytic activity. As shown in Figure 5-4, there is little effect of NO’ on the basal ECAR in these cells. Interestingly, the stimulatory effect of oligomycin and FCCP on ECAR described in Chapter 2 appears to be diminished following treatment with NO’. The mechanism by which this happens remains to be determined, however, there are some possible reasons for this difference. First, the measured ECAR depends on the ability to move glycolytic substrates into the cell, process them, and release the products as lactic acid. This must happen even while the ATP generating machinery of the mitochondrion is diminished (Figure 5-1) and oxygen consumption is decreased (Figure 5-3). All of the processes mentioned are energetically demanding, and while the cells may be able to compensate at baseline, the stimulatory effects of oligomycin and FCCP may result in a supramaximal demand on these import pathways. The decrease in OCR will likely yield lower ATP levels in the cell, and thus there will be less ATP available for substrate movement. A possible alternative could lie in total amount of mitochondria present in the cells. The data presented in Figures 5-1 and 5-2 suggest that there is decreased mitochondrial mass in these cells following NO treatment. If the increased ECAR that is normally seen in response to oligomycin and FCCP is a direct result of mitochondrial proton movement, and not increased glycolytic activity per se, then having less total mitochondrial mass could result in lowered responses to these compounds. Fur-
Figure 5-4: Chronic NO˙ treatment decreases glycolytic stimulation in response to oligomycin, FCCP, and antimycin A. Bovine aortic endothelial cells were seeded to 20,000 cells per well in Seahorse Biosciences V7 Tissue Culture Plates and allowed to grow for 24 hours. The cells were then treated with the indicated concentration of Deta NONOate for 16 hours. Following treatment, the cells were washed and switched to assay medium for 1 hour, and then the glycolytic activity was assessed using the measured ECAR (Panel A). Panel B shows the basal ECAR levels taken at the third baseline measurement. Data shown are the means ± sem. n≥3.
ther experiments are required to elucidate the exact cause of the difference in ECAR response to chronic NO‘.

To more directly examine the effects of chronic NO’ on mitochondrial function, the data shown in Figure 5-3 were examined as described in Chapter 2. These analyses are shown in Figure 5-5. First, the basal rate was plotted as a function of Deta NONOate concentration (Figure 5-5 Panel A). This demonstrates that chronic NO’ drastically decreases the amount of oxygen consumption occurring at baseline in these cells. Next, the ATP-Linked OCR was determined by subtracting the oligomycin-insensitive OCR from the basal OCR. This data is also plotted as a function of Deta NONOate concentration (Figure 5-5, Panel B). The remaining OCR from this calculation is ascribed to Proton Leak, and is shown in Figure 5-5, Panel C. As discussed previously, it is likely that this is an overestimate of the actual proton leak at baseline due to the hyperpolarization of the mitochondrial membrane in response to oligomycin; however, the comparison between the treatment groups yields valuable information regarding the effect of chronic NO’ treatment on mitochondrial membrane integrity. Notably, there is an upwards trend in the proton leak that occurs as a function of Deta NONOate concentration, however this measurement failed to reach statistical significance at most concentrations tested. Increased proton leak could indicate a decline in mitochondrial membrane integrity, and combined with the decrease in ATP-Linked OCR suggests that the mitochondria in these cells are less efficient. Lastly, the non-mitochondrial OCR is determined from the antimycin-A insensitive rate. Interestingly, this data (Figure 5-5, Panel D) shows a decrease in the amount of oxygen consumption occurring at sites other than Complex IV. While
Figure 5-5: Chronic nitric oxide treatment decreases total oxygen consumption at baseline. Bovine aortic endothelial cells were seeded at 20,000 cells per well in Seahorse Biosciences V7 Tissue Culture Plates and allowed to grow for 24 hours. The cells were then treated with the indicated concentration of Deta NONOate for 16 hours. Following treatment, the cells were switched to assay medium for 1 hour, and then the mitochondrial function assay was run. Panel A shows the baseline oxygen consumption rates plotted as a function of Deta NONOate concentration. The difference between the basal, and oligomycin-insensitive rates is plotted as the ATP-Linked OCR (B). The Proton Leak was calculated as the difference of the oligomycin-insensitive rate, and the antimycin A rate (C). The non-mitochondrial OCR is thus the antimycin A OCR alone. Data shown are the means ± sem. n≥3. *, p<0.05 vs. CTL.
we have not directly tested this, it is interesting to speculate that the decrease in non-mitochondrial OCR is directly attributable to a decrease in the total levels of Complex IV. The decrease in individual subunits of this Complex is shown in Figure 5-1 and discussed above. Ongoing experiments will attempt to analyze the direct reliance on Complex IV for ROS production and thus control of the antimycin-A insensitive rate, and this is discussed in further detail in Chapter 6.

**Apparent Respiratory State is Decreased by Nitric Oxide**

To next determine the apparent respiratory state in these cells following chronic treatment with the NO donor, the $\text{State}_{\text{apparent}}$ was calculated as described in Chapter 2. As shown in Figure 5-6, the $\text{State}_{\text{apparent}}$ decreases dramatically, and approaches State 3 respiration in a concentration dependent manner in response to Deta NONOate. This indicates that the cells are working closer to their maximal rate in response to chronic treatment with NO’. This conclusion is supported by the data shown in Figure 5-3 which demonstrates that the reserve capacity is greatly decreased after treatment with NO’, even when the FCCP stimulation is considered as a percentage of the basal OCR. This indicates that the cells are normally working at a rate closer to their maximum following NO’ treatment. Additionally, this suggests that the mitochondria are more uncoupled following NO’ treatment, as uncoupled mitochondria would be more similar to those in State 3. This idea is also supported by the data shown in Figure 5-2 that shows the membrane potential in response to NO’ to be decreased.
Figure 5-6: Apparent respiratory state decreases with chronic NO’ treatment. Apparent respiratory state was calculated as described in Chapter 2. Data shown are the means ± sem. n≥3. *, p<0.05 vs. 0μM group.
In addition, the degree to which the mitochondria are calling upon their spare capacity can also be calculated. This is done by determining the percentage of basal OCR as a function of the maximal, FCCP-stimulated OCR. This value is thus informative regarding the degree to which the mitochondria are utilizing their maximal capacity in these cells. As the mitochondria approach maximal workload, this value will approach 100%. As Figure 5-7 shows, chronic treatment with Deta NONOate increases the percent OCR utilization. Interestingly, this value does not appear to ever reach 100%, and instead plateaus at approximately 80% utilization. This implies that the mitochondria always have some reserve respiratory capacity under this stress. It is interesting to postulate that certain stressors may exceed this threshold, while others may only partially utilize the reserve capacity. This theory is tested in the next series of experiments, and will be discussed later in this chapter.

It is of interesting note that the two parameters of mitochondrial efficiency discussed in this section are similar, but distinctly different in the information that they provide regarding mitochondrial function. The State\textsubscript{apparent} value is a measure that was generated to describe the function of the mitochondria under conditions that are used to examine isolated mitochondria. Importantly, the State\textsubscript{apparent} yields little information regarding the integrity of the mitochondrial membrane, or of the magnitude of proton leak occurring in the cells, as the parameter used to determine this information (the oligomycin-insensitive rate) is subtracted out of all data points during the calculation (Equation 2-1, Chapter 2). The percent utilization, in contrast, is useful in that it takes into account the proton leak in the cells when determining the mitochondrial workload. Thus these
**Figure 5-7:** Chronic NO$^-$ treatment increases the percentage of maximal oxygen consumption used at baseline. The baseline rates shown in Figure 5-3 were divided by the FCCP-stimulated oxygen consumption rates to calculate the percent of maximal OCR. Data shown are the means ± sem. n≥3. *, p<0.05 vs. the 0μM group.
two pieces of data yield information related to two different indices of mitochondrial function, both of which are valuable.

**Coupling Efficiency is not Changed in Response to NO⁻**

To next examine the extent to which the mitochondria are coupling oxygen consumption to ATP production, the coupling efficiency was calculated as described in Chapter 2. As shown in Figure 5-8, BAEC treated with Deta NONOate for 16 hours have little change in their overall coupling efficiency. This parameter is calculated by determining the amount of oxygen consumption that is linked to ATP production as a percentage of the basal oxygen consumption that is mitochondrially derived. This result implies that chronic treatment with NO is not changing the efficiency of the mitochondria, even though the apparent respiratory state, and percentage of maximal OCR utilization have increased. Importantly, this information yields a third view of mitochondrial function in these cells in response to NO that is distinctly different from the data yielded in the calculations discussed above.

**Chronic NO⁻ Does not Alter the Acute Response to DMNQ**

As shown in Chapter 3, the redox cycling compound 2,3-dimethoxy-1,4-napthoquinone (DMNQ) is able to increase OCR in a flavin-dependent manner, and also decreases the cellular response to FCCP. This results in a diminished reserve capacity, which could impair the ability of a cell to respond to stress. The effects of chronic administration of NO⁻ on the response of the cells to DMNQ was examined in the next series of experiments. BAEC were seeded to 20,000 cells/well as in the above experiments
Figure 5-8: Coupling efficiency is only minimally changed in response to chronic NO\textsuperscript{+} treatment. Coupling efficiency was calculated by determining the percentage of basal OCR that is due to ATP-linked O\textsubscript{2} consumption. Data shown are the means ± sem. n\geq3. *, p<0.05 vs. the 0\mu M group.
and allowed to incubate for 24 h. The cells were then treated with Deta NONOate to a final concentration of 250 μM. The plate was again allowed to incubate for 16 h and the media was then changed to unbuffered assay media. The NO’ donor was washed out with this step. The cells were then incubated in a non-CO2 incubator for 1 h prior to the start of the experiment. Three basal measurements of oxygen consumption were then taken, and this was followed by injection of DMNQ to 7.5 μM. The cells were allowed to incubate with DMNQ for 1 h and three further measurements of OCR and ECAR were made. Following these measurements, mitochondrial function was assessed using the mitochondrial function assay described in Chapter 2. The cells were treated sequentially with oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) with a measurement of OCR and ECAR following each measurement. The results of this experiment are shown in Figure 5-9. This figure demonstrates the effects of acute DMNQ treatment, and compares the effects of this treatment when on the background of chronic NO’ administration. Consistent with the results reported in Chapter 3 which used DMNQ in a similar experimental model, DMNQ on its own stimulates OCR in these cells. Additionally, the cells receiving the chronic NO’ treatment exhibit a large inhibition of basal oxygen consumption that persists for the duration of the experiment in concordance with the data shown in Figures 5-3 and 5-5 (data not shown). Interestingly, the cellular response to DMNQ does not appear to be impacted by the chronic NO’ treatment when the data are plotted as a percentage of the original baseline (Figure 5-9). This conclusion differs slightly when the data are not corrected for the initial difference in basal oxygen consumption. Figure 5-10 Panel A demonstrates that following chronic NO’ treatment, cells subsequently treated with DMNQ no longer respond to FCCP, and in fact have lower
Figure 5-9: Chronic treatment with NO\textsuperscript{•} does not alter the response to acute DMNQ injection. BAEC were seeded to 20,000 cells/well in Seahorse Bioscience V7 Tissue Culture Plates. The cells were allowed to grow for 24 h, and then indicated groups were treated with 250 μM Deta NONOate. The cells were allowed to incubate for 16 h and were then washed with assay media for 1 h prior to the start of the experiment. Three baseline measurements of OCR and ECAR were made before injection of 7.5 μM DMNQ where indicated. The cells were allowed to incubate for 1 h and three further measurements of OCR and ECAR were made. This was followed by sequential injection of oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) with a measurement of OCR and ECAR between each injection as indicated. Data were calculated as the percentage of the original basal OCR measurement, and are shown as the means ± sem. n\geq3 per group. Statistical indicators are omitted from this graph for visual clarity.
Figure 5-10: Chronic administration of NO\textsuperscript{–} suppresses the response to DMNQ and FCCP. BAEC were seeded to 20,000 cells/well in Seahorse Bioscience V7 Tissue Culture Plates. The cells were allowed to grow for 24 h, and then indicated groups were treated with 250 μM Deta NONOate. The cells were allowed to incubate for 16 h and were then washed with assay media for 1 h prior to the start of the experiment. After 3 baseline measurements, DMNQ was injected to 7.5 μM and allowed to incubate for 1 h. The cells were then examined using the mitochondrial function assay as described. Panel A shows the response to DMNQ taken as the third basal OCR measurement following DMNQ injection. Additionally, the maximal OCR is plotted indicating the level of maximal oxygen consumption possible at Complex IV. Panel B depicts the reserve capacity calculated from the experiment. Data shown are the means ± sem. *, p≤0.05 vs. control maximal OCR. #, p≤0.05 vs. control basal OCR. †, p≤0.05 vs. control reserve capacity. §, p≤0.05 vs. Deta NONOate reserve capacity. n≥3 per group.
overall oxygen consumption than the DMNQ-only treated group. As shown in Figure 5-10 Panel B, this results in a decreased reserve capacity in both the DMNQ and NO + DMNQ treatment groups. This experiment indicates that these treatment groups actually have a negative reserve capacity. Importantly, this likely indicates that the DMNQ treatment is inhibiting oxygen utilization in the cell through an as yet undefined mechanism. It is of important note that the total oxygen consumption is far lower in the cells that have received chronic NO’ prior to the DMNQ challenge. Panel A of Figure 5-10 demonstrates that the total OCR in these cells at baseline is much lower than the control cells. This could lead to a deficiency in the ability to respond to a secondary stressor, such as DMNQ, and thus result in a larger impairment of cellular oxygen consumption over time. The possibility that chronic NO’ treatment is responsible for this shift in susceptibility is examined more thoroughly in the next series of experiments.

Chronic NO’ Sensitizes BAEC to Apoptosis Induced by DMNQ

In order to determine the consequence of treatment with sequential NO’ and DMNQ, induction of cell death was next examined in this model system. In the first series of experiments, BAEC were treated with the NO’ donor Deta NONOate for 16 h at 250 µM. After this, the cells were washed twice with warm medium, and treated with DMNQ at 7.5 µM for an additional 8 h. Representative photomicrographs of these cells are shown in Figure 5-11. Interestingly, neither the NO’ donor nor the DMNQ were toxic at these concentrations, however, when given in combination, a large number of the cells appear to be dying, and many have detached from the plate.
Figure 5-11: Cytotoxicity of chronic NO$^-$ and DMNQ is markedly increased after sequential treatment with these compounds. BAEC seeded into 6-well plates were treated with the NO donor Deta NONOate at 500 μM for 16 h in complete DMEM. The cells were then washed twice with warmed media and treated with DMNQ at 7.5 μM for an additional 8 h. Photomicrographs were then acquired. Representative images are shown.
Figure 5-12: Chronic treatment with NO⁻ sensitizes endothelial cells to apoptosis induced by DMNQ. BAEC seeded into 6-well plates were treated with the NO⁻ donor Deta NONOate at 500 μM for 16 h in complete DMEM. The cells were then washed twice with warmed media and treated with DMNQ at 7.5 μM for an additional 8 h. Some treatment groups received staurosporine (1 μM) as a positive control for the induction of apoptosis. The cells were then lysed in a CHAPS-based lysis buffer and probed for Cleaved Caspase 3 and 9. Data shown are representative images. n=3 per group.
This experiment was followed by an examination of the cell death pathways involved. Cells treated in the manner described above were lysed and harvested, and then examined for activation of caspases. Caspase cleavage indicates activation of these enzymes, and is a prototypical hallmark of apoptotic cell death. As Figure 5-12 shows, both Cleaved Caspase 3 and 9 are detectable following the sequential treatment with the NO` donor and DMNQ, but not as a result of either treatment alone. This likely indicates that the cells are undergoing a mitochondrial-dependent apoptotic cell death in response to NO` and DMNQ. The mechanism of apoptosis induction in this model system remains unclear, however there are several potential pathways that could be implicated in this process. These will be discussed in further detail below.

**DISCUSSION**

The data in this chapter demonstrate a third mechanism by which cellular bioenergetics can be affected by treatment with pathologically relevant compounds. In the previous chapters, two different treatment schemes were used to examine whole-cell bioenergetic state in response to stressors. Specifically, the response to acute administration of nitric oxide was examined in an endothelial cell line in Chapter 3, and induction of oxygen consumption was analyzed in response to an acute addition of 4-hydroxynonenal using myocytes in Chapter 4. While these compounds have known physiological significance, and are widely accepted as potential mediators of acute vascular stress, the response to chronic treatment of a compound on cellular bioenergetics had not been examined. Importantly, the data presented in this section defined the cellular response to chronic NO` administration, and demonstrated for the first time a large decrease in the
basal OCR in response to this compound. Persistent inhibition of OCR was seen with concentrations of the NO’ donor as low as 50 μM. Based on the half-life of this donor, this is expected to release approximately 100 nmol of NO’ at steady-state over the course of the 16 h treatment [137]. Taking into account some uptake and metabolism of the NO’, this release of NO’ is in the low pathological range. Importantly, the endothelium is exposed to large concentrations of NO’ produced by iNOS in macrophages during the pathogenesis of atherosclerosis.

The data presented here demonstrate for the first time the effects of chronic NO’ administration on cellular oxygen consumption in endothelial cells. Previously, our laboratory demonstrated that chronic NO’ treatment resulted in a decrease in mitochondrial complex levels [29]. Several groups of researchers have proposed that long-term treatment with NO’ decreases mitochondrial oxygen consumption [67, 137, 224]. However, these authors also indicate that there is some stimulation of oxygen consumption over time with administration of an NO’ donor, which may be dose dependent [137]. Furthermore, these effects have been attributed to the formation of an S-nitrosothiol on Complex I [67, 238], though the mechanism of S-nitrosothiol formation from this NO’ donor remains unclear. It is unlikely that S-nitrosation of Complex I plays a role in the inhibition of oxygen consumption shown in this chapter, as it has been reported that the NO’ donor used (Deta NONOate) is unable to increase S-nitrosothiol formation on its own [239]. Further, we have used a sensitive method for the detection of protein thiols based on the reactivity of iodoacetamide [240], and have seen no effect following chronic treatment with Deta NONOate (data not shown). Together, these findings indicate that
these data constitute the first description of a long-term non-reversible inhibition of oxygen consumption in adherent endothelial cells by nitric oxide.

**Defining a Threshold for the Detrimental Effects of NO**

The data presented in this chapter support the idea that cells can maintain inhibition of a large amount of oxygen consumption without obvious detriment to the overall cellular function. In agreement with this, cells treated with as high as 500 μM Deta NONOate display a large inhibition of OCR (Figure 5-3, 5-5), but virtually no cell death (Figure 5-11, 5-12). Furthermore, it is generally accepted that mitochondrial diseases that result from respiratory defects are seen first in neuronal tissues and in skeletal muscle, as these tissues have a high dependence on mitochondrial oxidative phosphorylation [241, 242]. These data suggest that chronic NO inhibits oxygen consumption, and results in two distinct phenotypes. Initially, there is a large decrease in the OCR in cells treated with the NO donor. Additionally, the reserve capacity is significantly decreased in these cells, and this appears to concentration dependent (Figure 5-3 Panel B). This suggests that the inhibition of respiration is causing an increase in the utilization of the cellular reserve capacity in these cells, and this drives the State towards 3.0, akin to maximal respiration. The increased cell death seen in response to a challenge with DMNQ suggests that the secondary oxidative stress generated by this compound is normally compensated with by utilizing the reserve capacity in these cells. Figure 5-13 shows this concept schematically. In the absence of NO, the cells have a large reserve capacity such that Basal OCR is maintained throughout a respiratory stress. The cells are able to compensate by using their reserve capacity which maintains OCR up to a thresh-
Figure 5-13: An altered threshold sensitizes cells to stress. This figure represents a model for the effects of NO· in sensitizing cells to oxidative stress. The data presented in this chapter suggest that NO· alters the threshold above which the cells cannot sustain oxygen utilization in response to DMNQ. This indicates that the concentration that would elicit toxicity in response to DMNQ would be lower than in control cells.
old where this can no longer be sustained. Above this threshold, the OCR decreases rapidly, and the cells are unable to maintain cellular function. Panel B of this figure displays the hypothesized effect of chronic NO\(^-\) treatment. The data presented in this chapter suggest that one mechanism whereby NO\(^-\) sensitizes cells to DMNQ-induced cytotoxicity is through altering the threshold where the cells respond to stress. Because chronic NO\(^-\) treatment decreases the reserve capacity, it is hypothesized that the cells cannot maintain function with increased stress. This would result in lower concentrations of a stressor causing cytotoxicity, and thus the threshold exhibits a left-shift.

**Summary**

The data in this chapter provide the first indication of persistent inhibition of oxygen consumption by nitric oxide in endothelial cells. These data build upon our previous findings that demonstrate a decrease in mitochondrial respiratory chain complex activities [29]. Our data further these studies by demonstrating a decrease in individual subunits of Cytochrome c Oxidase, the enzyme responsible for oxygen consumption in intact mitochondria (Figure 5-1). This led to the examination of oxygen consumption following the NO\(^-\) treatment, which we show here to be dramatically decreased (Figure 5-3). Lastly, we determined the respiratory State\(_{\text{apparent}}\) in these cells following chronic NO\(^-\) treatment and found it to be decreased in a concentration-dependent manner. These data demonstrate that exogenous administration of NO\(^-\) impacts on cellular bioenergetic function, and could lead to susceptibility to secondary stressors as shown in Figures 5-11 and 5-12. Importantly, these data raise the possibility of a threshold that defines the cellular re-
sponse to a stressor, and indicate that nitric oxide modulates this threshold to sensitize endothelial cells to stress.
CHAPTER 6

DISCUSSION

The data in this thesis describe a role for mitochondrial bioenergetics in several model systems. To do this, we adopted a novel technology that allowed the simultaneous quantification of multiple pathways of bioenergetics in intact cells. These data demonstrate for the first time a view of the bioenergetic response in intact cells. In Chapter 2 of this thesis, the methods for determining the functional consequence of treatment with a compound of interest were discussed. These novel techniques allowed us to describe the effects of acute nitric oxide (NO\textsuperscript{-}) on endothelial cell function (Chapter 3), the effects of 4-hydroxynonenal (HNE) on primary isolated neonatal rat ventricular myocytes (Chapter 4), and lastly the effects of chronic NO\textsuperscript{-} on mitochondrial function which persists beyond treatment with the NO\textsuperscript{-} donor (Chapter 5).

While we have focused on NO\textsuperscript{-} and HNE in the cardiovascular system, the data discussed herein have much broader applicability to the study of disease. HNE is known to increase in several disease states including chronic inflammation, heart disease, and kidney failure [92, 147, 243]. Furthermore, NO\textsuperscript{-} is a potent mediator of cellular function that may be involved in liver injury [244, 245], renal dysfunction [246-248], neurodegeneration [249] and other diseases. This chapter will first discuss the directions future experiments will take in light of the major findings presented in the previous chapters.
Next, implications of the work described in this thesis will be discussed, with a discussion of the importance of the reserve capacity in the models presented here. Finally, a view of what is to come in the field of mitochondrial bioenergetics with respect to the study of disease will be discussed.

**PROBING THE STRUCTURE/FUNCTION RELATIONSHIP OF UBIQUINONE CYTOCHROME C OXIDOREDUCTASE AND CYTOCHROME C OXIDASE**

As discussed in Chapter 1, defects in mitochondrially-encoded genes often result in disease. Deletions or mutations in the mitochondrial DNA often result in loss of function and decreased respiratory capacity in many tissues, though muscle and neurons are most susceptible. This results in diseases such as Leber’s Hereditary Optic Neuropathy (LHON) and Mitochondrial Encephalomyopathy Lactic Acidosis with Stroke-Like Episodes (MELAS), the two most common diseases associated with mitochondrial dysfunction [23, 24, 250]. It is only now becoming appreciated that nuclear-encoded subunits of COX and UQCR play a role in the development of many diseases [251, 252]. This is a result of several major discoveries surrounding the cross-talk between the nuclear and mitochondrial genomes [253, 254], and how defects in this process can lead to errors in the production and assembly of nuclear-encoded gene products necessary for enzyme function. These include the defects related to assembly of intact Complex I [241], Complex III [242, 255, 256], and Complex IV [235]. Additionally, recent evidence suggests that assembly defects in Complex IV may affect Complex I stability [257], and may furthermore lead to nuclear genome instability [258]. Together, these studies highlight the importance of the nuclear-encoded subunits of these complexes in controlling cellular function.
While the structure of cytochrome c oxidase (COX) was solved in 1996 [236] and ubiquinone cytochrome c oxidoreductase (UQCR) in 1997 [259], the role for some of the subunits comprising these enzymes remains unclear. In mammalian COX, only 3 of the 13 subunits that comprise the holoenzyme are encoded by the mitochondria. In UQCR, merely 1 of the 11 subunits is mitochondrially encoded. In the case of COX, the main catalytic activity of the enzyme can be accomplished by just the catalytic core of the 3 mitochondrially encoded proteins. This raises the question of the role the other 10 nuclear-encoded proteins are playing. While deletion of UQCR subunits does not allow this level of activity, it is clear that the nuclear encoded proteins play a secondary role to the redox center-containing mitochondrially encoded cytochrome b subunit. While the function of the mitochondrially encoded proteins has been extensively researched, less attention has been paid to the nuclear encoded subunits. Recent research has implicated these nuclear encoded proteins in the control of proton pumping [260, 261], binding of cytochrome c to Complex IV [262-264], and holoenzyme structure [237, 261, 265-268]. All of these processes are directly related to mitochondrial O$_2$ consumption, and alterations could thus impact on the level or efficiency of this process [269, 270].

Extending these findings are studies suggesting that nuclear encoded subunits of COX are responsive to cellular oxygen concentration in Dictyostelium discoideum [271], and more recently in mice [11]. These subunits have been proposed as the cellular oxygen “molecular switch” that senses and responds to hypoxia. Moreover, there is an increasing necessity to examine the role of individual nuclear-encoded subunits in total mitochondrial function to address the question of the role these subunits play in intact sys-
tems. Towards this goal, we have adopted an siRNA approach to selectively knock down nuclear-encoded subunits of mitochondrial complexes in cell culture.

**Generating a Complex IV Specific Protein Knockdown**

In the first series of experiments, an siRNA approach was used to knockdown the levels of a nuclear-encoded subunit of Cytochrome c Oxidase (COX). These experiments were performed in Human Umbilical Vein Endothelial Cells (HUVEC) due to the need for a human gene sequence on which to base the siRNA construct design. Commercially-available siRNA constructs directed against Subunit IV of COX were transfected into these cells using Lipofectamine 2000. The cells were allowed to incubate with this transfection in antibiotic-free medium for 8 hours, and then the media was changed and the cells were allowed to grow for an additional 40 hours. The cells were then harvested, and protein levels of COX IV were determined by Western blot and quantified by densitometry (Figure 6-1). As shown in this figure, the protein levels of COX IV were depleted greater than 60% 48 h post-transfection. Interestingly, we found that the protein levels of COX Subunit I were also decreased by 50% (Figure 6-1 Panel B). Because COX I is mitochondrially encoded, there is little possibility of cross talk with the degradation system for nuclear-encoded proteins. These data then indicate that the decrease in COX I levels seen after treatment with COX IV siRNA could be related to a decrease in enzyme assembly or stability.
Figure 6-1: Knockdown of Cytochrome c Oxidase Subunit IV using siRNA.
HUVEC were left untreated or treated with non-silencing siRNA, or siRNA directed against Cytochrome c Oxidase Subunit IV (COX IV) for 8 h. The media was changed, and the cells were allowed to grow for an additional 40 h. The cells were then harvested, and probed for COX IV and I levels by Western blotting (A). Representative western blots are shown. Panel B shows the quantitation of these blots. Data are the means ± sd. n=3. *, p<0.05 vs. untreated.
Depletion of One Nuclear-Encoded Subunit of Cytochrome c Oxidase Affects Stability of the Entire Complex

To investigate the possibility of altered Complex IV stability in intact cells, we employed the MDA-MB-231 breast adenocarcinoma cell line which contains large amounts of mitochondria, and can be easily expanded. Because an examination of the levels of intact COX and UQCR requires native protein analysis, the use of a cell line with a high mitochondrial content was crucial to our initial studies. In the next series of experiments, the MDA-MB-231 cells were transfected with siRNA constructs directed against either Subunit IV of COX or Core 2 of UQCR, both of which are nuclear encoded. Five 100 mm dishes were harvested for each treatment group, and subjected to gentle homogenization and differential centrifugation to isolate a crude mitochondrial pellet. These mitochondria were then solubilized using lauryl maltoside, a mild detergent that disrupts the mitochondrial membrane, but leaves the respiratory chain complexes intact. These samples were then run on a 4-15% gradient Blue Native Gel to resolve individual complexes. The gel was then reduced using β-mercaptoethanol to disrupt the subunits of each complex, but allow them to remain at the proper molecular weight for the intact complex. The gel was then transferred to a nitrocellulose membrane, and probed by Western blot using a cocktail of antibodies directed against Complex I, and non-targeted subunits of Complex III and IV. The resulting blot is shown in Figure 6-2 Panel A. This technique allows for the determination of total levels of Complexes I, III, and IV, and also gives an indication of the size of the complex. If only an individual subunit is lost due to the siRNA knockdown and the complex is still able to assemble and remain stable, it will run as a lower molecular weight band on this native gel. If however the complex is not stable in the absence of the targeted subunit, it is likely that there will be a decrease in
Figure 6-2: Total complex loss is achieved through selective knockdown of nuclear encoded subunits of Cytochrome c Oxidase or Ubiquinone Cytochrome c Oxidoreductase. MB231 breast cancer cells were grown in 100mm dishes, and were left untreated, or treated with non-silencing siRNA, or siRNA constructs directed against Cytochrome c Oxidase Subunit IV or Ubiquinone Cytochrome c Oxidoreductase Core 2. The cells were treated for 8 h, and then the media was changed. 40 h later, the cells were harvested, crude mitochondrial pellets were collected by differential centrifugation, and the samples were subjected to Blue-Native Gel Electrophoresis. The gel was then reduced using β-mercaptoethanol, and the proteins transferred to nitrocellulose membrane. The levels of Complex I, II, and IV were then detected using antibodies directed against subunits not targeted for degradation. Data shown are the fold change in each complex level versus the untreated sample.
the total band intensity and the band will appear at the expected, full molecular weight. As shown in Figure 6-2 Panel A, cells treated with either the COX IV or UQCRC2 directed siRNA demonstrated a decrease in the total levels of the intact complex that was targeted. Densitometric analysis of this blot is shown in Figure 6-2 Panel B. Interestingly, the bands that are present representing either Complex IV or Complex III do not appear to have a shifted molecular weight. This indicates that there is a loss in total complex level when either of these nuclear encoded subunits are targeted, and not assembly of complexes with abnormal subunit stoichiometry.

**Knockdown of Complexes III or IV Changes Cellular Bioenergetic Status**

The above described experiments were performed to more clearly understand the structure/function relationship of Complexes III and IV as related to cellular oxygen consumption. In the next set of experiments, basal oxygen consumption was examined in HUVEC treated with siRNA constructs directed against the subunits already mentioned, as well as Complex III Rieske Iron Sulfur Protein (UQCRFS) or Complex IV Subunit Vb (COX Vb). Similar to the experiments above, preliminary data suggested that these subunits may also have a role in maintaining holoenzyme function and integrity. As shown in Figure 6-3, siRNA knockdown of COX IV, Vb, or UQCRC2 had little impact on the basal OCR. Interestingly, there was a strong decrease in the OCR when cells were treated with siRNA directed against UQCRFS. The measured ECAR increased in all groups, but this increase was the strongest in the cells treated with the UQCRFS siRNA, inversely mirroring the results obtained with the OCR values (Figure 6-3 Panel B). When OCR and ECAR are plotted against each other (Figure 6-3 Panel C) it is evident that knock-
Figure 6-3: Knockdown of Electron Transport Chain protein subunits shifts the metabolic profile of HUVEC. HUVEC were treated with the indicated siRNA construct for 8 h, the media was changed, and the cells were allowed to grow for an additional 40 h. Mitochondrial oxygen consumption (A) and extracellular acidification (B) were then determined. The comparison of these two parameters indicates the shift from aerobic metabolism to a more glycolytic phenotype (C). Data shown are the means ± sem. n≥3 per group. *, p<0.05 vs. Control.
down of UQCRFS has a dramatic impact on the energetic phenotype of these cells. Of the cells lines described in Chapter 2 which we have now characterized, HUVEC rank among the lowest in overall metabolic activity. Further studies are required to confirm the results discussed above, and these experiments will be performed using a cell type with a higher bioenergetic demand to increase the sensitivity of our results. Taken together, these data point towards an important role for individual nuclear encoded subunits of the respiratory chain in controlling the cellular bioenergetic status. Future studies will examine the specificity of this response, and will seek to determine the role of each subunit of these large mitochondrial complexes in the control of mitochondrial oxygen consumption.

**STIMULATING MITOCHONDRIAL FUNCTION WITH POST-TRANSLATIONAL PROTEIN-O-GlcNAc ADDUCTS**

Post-translational protein modifications are one potential mechanism whereby cells could be protected from the oxidant stressors discussed in the previous chapters. Several mechanisms have been proposed to accomplish this, but many researchers have suggested that certain non-deleterious post-translational protein modifications may serve as a “cap” to protect against other harmful modifications. One proposed example of this is the generation of S-nitrosothiols on cysteine thiols to protect against the deleterious effects of reactive aldehydes such as HNE [272]. Another modification which has been suggested to play a role in this protection is the addition of O-GlcNAc to proteins. These modifications consist of the O-linked addition of a glucosamine sugar moiety to serine or threonine residues on proteins. Though the exact mechanism of protection is unknown, increased transient protein-O-GlcNAcation has been reported to protect from ische-
mia/reperfusion injury [273, 274], acute hydrogen peroxide treatment [108], and endoluminal injury [275]. We thus sought to examine the role of protein-\(O\)-GlcNAc adducts on cellular bioenergetic status, and examine the potential for increased bioenergetic reserve as a mechanism contributing to protection from vascular injury.

Figure 6-4 shows the predominant metabolic pathways involved in the formation of protein-\(O\)-GlcNAc adducts. Glucosamine is converted to UDP-GlcNAc, which is a substrate for L-glutamine-D-fructose-6-phosphate amidotransferase (GFAT). UDP-GlcNAc is then used as a substrate for \(O\)-GlcNAc Transferase (OGT) in the addition of the GlcNAc sugar to serine and threonine residues on proteins. As mentioned above, this post-translational modification is dynamic, and can be removed through the action of \(O\)-GlcNAcase (OGA). To determine the role of this pathway on cellular processes, several commercially available compounds have been used. Exogenous administration of glucosamine works to increase the amount of UDP-GlcNAc available for conjugation to proteins. \(O\)-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc) acts to inhibit OGA and thereby decrease the removal step of this process. NAG-bt works in a similar manner to PUGNAc, though has been reported to have a much greater specificity for OGA than PUGNAc. The NAG-bt compound is not available commercially, and its use here is through a generous gift from Dr. John Chatham at the University of Alabama at Birmingham.

In this series of experiments, neonatal rat ventricular myocytes (NRVM) were treated with the three compounds mentioned above that are known to increase protein \(O\)-
Figure 6-4: Production of O-GlcNAc via the Hexosamine Biosynthetic Pathway. O-GlcNAc protein adducts are formed through the action of the hexosamine biosynthetic pathway. UDP-GlcNAc, the substrate for O-GlcNAc transferase (OGT) can be formed endogenously through the action of glucosamine:fructose-6-phosphate amidotransferase (GFAT), or exogenously through conversion of glucosamine to glucosamine-6-phosphate by hexokinase. OGT is then responsible for the addition of O-GlcNAc to serine or threonine residues on proteins. O-GlcNAc adducts can then be removed by O-GlcNAcase (OGA). O-GlcNAc levels can thus be increased pharmacologically through the application of inhibitors of OGA. PUGNAc and NAG-bt are two inhibitors used in this study to increase protein-O-GlcNAc adducts. This enzymatic system allows for dynamic control of protein-O-GlcNAc modifications in response to acute stressors.
GlcNAc adducts. The cells received either 5 mM glucosamine, 100 μM PUGNAc, or 100 μM NAG-bt for 4 h. The cells were then washed with assay media for 1 h, and mitochondrial function was assessed using the assay described in Chapter 2. The effects of each of these compounds on OCR are shown in Figure 6-5 Panel A. All of the tested compounds demonstrated an increasing trend in every parameter tested, but PUGNAc had a statistically significant increase at all measurements. This treatment also resulted in an increase in the reserve capacity in these cells, evident from the increase in the FCCP-induced OCR versus control cells. In a parallel experiment, NRVM treated as above were harvested following the 1 h assay medium wash to examine cellular O-GlcNAc-protein adduct levels. These cells were lysed using RIPA buffer, and equal protein was loaded onto an SDS-PAGE gel. The proteins were separated, and Western blotted for protein-O-GlcNAc adducts using the CTD 110.6 antibody. As shown in Figure 6-5 Panel B, PUGNAc and NAG-bt treatment for 4 h was able to increase the protein-O-GlcNAc adducts in these cells. Interestingly, glucosamine treatment for the same period of time did not increase detectable protein-O-GlcNAc adducts. It is expected that this difference is due to the difference in mechanism of O-GlcNAc stimulation. As mentioned above, glucosamine will increase substrate for UDP-GlcNAc production, but as these post-translational modifications are dynamic, they could be removed during the 1 h media change in the protocol. Importantly, treatment with 5mM glucosamine has previously been demonstrated to increase protein O-GlcNAc adducts in these cells [108]. In contrast, PUGNAc and NAG-bt both work to inhibit OGA, the enzyme responsible for removing O-GlcNAc adducts. This likely indicates that the adducts stimulated by the
Figure 6-5: Pharmacologically increasing O-GlcNAC yields higher OCRs as well as increased O-GlcNAC protein modification. NRVM were treated with glucosamine (5mM), PUGNAc (100μM), or NAG-BT (100μM) for 4h. The cells were then either probed for mitochondrial function (A), or O-GlcNAC protein modification. Protein O-GlcNAC modifications were identified using the CTD 110.6 antibody (B) and quantified (C). Data shown are the means ± sem. n=5 per group, *,p≤0.05.
treatment with PUGNAc and NAG-bt were not removed during the course of the experiment, and are still present to be detected by Western blot.

Together these data suggest that post-translational modifications of proteins by O-GlcNAcation may be an effective strategy for improving the cellular respiratory status and increasing the reserve capacity. Further experiments are needed to determine is this stimulation is specific for O-GlcNAcation or if other post-translational modifications are capable of modulating cellular bioenergetics. Furthermore, a determination of whether the modifications are beneficial is also required. As mentioned above, transient O-GlcNAc stimulation during hypoxia and reoxygenation in cell culture has been demonstrated to be protective. It remains to be determined if this protection is due to stimulated bioenergetic reserve capacity.

THE IMPORTANCE OF THE RESERVE CAPACITY IN CELLULAR RESPONSES TO STRESS

The data discussed thus far in this thesis constitute a new view of the reserve capacity in intact cell culture systems. While knowledge of a high energy phosphate reserve capacity has existed for some time now, little is known about the use and utility of this reserve capacity in response to stressors or stimuli. Here we have shown that the reserve capacity is depleted in response to several stressors with relevance to cardiovascular disease. This is directly related to our understanding of the failure of energy supply in the pathogenesis of several cardiovascular disease states. As mentioned in previous chapters, depression in the amount of ATP available in endothelial cells is one possible contributor to endothelial dysfunction. Similarly, inefficient cardiac mitochondria are
thought to underlie myocardial pathologies during heart failure [207]. This concept agrees with experimental evidence suggesting that respiratory chain activity is decreased in the diseased heart [161, 162]. Our data describe a new role for the reserve capacity in controlling the response of the cell to multiple stress stimuli from a bioenergetic perspective. Interestingly, all three datasets presented in this thesis result in a decrease in the reserve capacity parameter of oxygen consumption, however the data also show that this happens through three distinct mechanisms. The major findings of this thesis with respect to reserve capacity are discussed in depth below.

**Modulation of the Reserve Capacity by Acute Nitric Oxide Treatment**

In Chapter 3, the bioenergetic response to acute treatment with the nitric oxide donor Deta NONOate was examined. This chapter clearly showed that treatment with NO\(^-\) resulted in a decrease in the FCCP-stimulated OCR. As a consequence of this inhibition, we also showed that the reserve capacity was depleted following acute nitric oxide treatment. Importantly, this deficiency was not carried into the basal OCR. As indicated in Figure 2-5, the reserve capacity is directly related to the FCCP-stimulated OCR. This then defines one possible scenario for the depletion of the reserve capacity. A decrease in FCCP-stimulated OCR, while maintaining basal oxygen consumption thus results in a lack of ability to respond to secondary stimuli. The next set of experiments in Chapter 3 were then designed to test this hypothesis. The next set of data demonstrate that cells treated with a combination of the NO\(^-\) donor and an ROS generating compound, DMNQ, were more susceptible to cell death. Indeed, these cells had a complete loss of the reserve capacity, and became unresponsive to FCCP treatment. Together these data suggest that
depletion of the reserve capacity through inhibition of OCR stimulation is one important method for depleting the reserve capacity, and that this can impact on cellular survival.

These data agree with previous experiments which show that NO’ inhibits OCR much more thoroughly when mitochondria are in active turnover. Importantly, this suggests that NO’ may so little to impact on the basal OCR under normal physiological conditions. Furthermore, these data suggest that NO’ could be playing a strong role in the control of OCR during pathologic states. This could occur when respiratory rate is increased due to protein damage or chronic overstimulation. The resultant inefficiency of oxygen consumption leads to increased turnover and thus heightened sensitivity to inhibition by NO’. This state is potentially present following endoluminal injury, or in endothelial dysfunction concurrent with atherosclerosis. Importantly, we have discussed this inhibition of the reserve capacity as a negative effect of acute NO’ treatment; however, NO’ may be serving to govern the rate of oxygen consumption in the above-mentioned conditions. Uncontrolled oxygen consumption could theoretically damage surrounding tissues by unnecessarily depleting the oxygen delivered to a given tissue. Increased sensitivity to NO’ in these damaged tissues may be present as a control mechanism thereby limiting damage to a smaller area. Finally, these data are consistent with a large role for NO’ control of respiration during hypoxia. As oxygen levels decrease, interactions of NO’ with Cytochrome c Oxidase result in inhibition of the enzyme, even while having little effect on basal OCR. This potentially explains a mechanism whereby NO’ acts to extend oxygen gradients in tissues subject to hypoxia.
**Stimulation of OCR by HNE Leads to Reserve Capacity Utilization**

In Chapter 4 a new model system was introduced to demonstrate the crucial nature of the reserve capacity in an excitable cell type. Isolated neonatal rat ventricular myocytes (NRVM) were cultured and examined for their response to the reactive aldehyde 4-hydroxynonenal (HNE). In this series of experiments, NRVM demonstrated a strong increase in OCR in response to HNE treatment. We found this stimulation to be dose dependent and biphasic. The stimulation by HNE decreased over time, and eventually became inhibitory. At lower concentrations of HNE (5-10 μM) there was no effect seen on the FCCP-stimulated OCR. This result is in contrast to the data shown in Chapter 3 and discussed above. This stimulation of OCR resulted in a “consumption” of the reserve capacity. That is, the cells increased their OCR at baseline towards the maximal OCR achieved by FCCP. The net effect of this reserve capacity utilization mirrors the effect of the acute NO’ treatment; however, the mechanism differs greatly. The data presented in this chapter show a large decrease in the remaining reserve capacity that is available to the cell. In this set of experiments, higher concentrations of HNE (20-30 μM) resulted in a complete loss of the reserve capacity and were thus associated with cell death as shown in Figure 4-4.

These data are consistent with a decrease in efficiency during heart failure and a role for HNE in mediating this inefficiency. Several research groups have reported interactions of HNE with VDAC, leading to increased proton leak [93, 94]. While these findings were made in isolated mitochondria, the data presented here demonstrate concordance in intact cell systems. Together with the stimulatory effects of HNE on ECAR (Fig-
Chronic Nitric Oxide Treatment Depletes Reserve Capacity in Endothelial Cells

Following from the data shown in Chapter 3, the response of endothelial cells to chronic treatment with NO’ was examined in Chapter 5. The data presented in this chapter demonstrated a decrease in the reserve capacity by a third, distinct mechanism. In contrast to the data shown with acute injections of NO’, this series of experiments employed a model whereby BAEC were treated with an NO’ donor for 16 hours. The donor compound was then removed from the media with a change to assay media. This experimental design allowed for the determination of the long-term effects of NO’ on these cells without any interference of the acute effects which were already shown to change mitochondrial function (Chapter 3). The result of this treatment was a large decrease in both basal oxygen consumption, and a decrease in the reserve capacity. The change in reserve capacity was measured by two distinct methods, and was found to be decreased in both. First, the absolute amount of O₂ that could be stimulated by FCCP was determined, and found to be decreased following NO’ treatment. In addition, the reserve capacity was determined as a percentage of the basal oxygen consumption and was found to be decreased as well. While not directly tested, these data imply that overall mitochondrial mass may be affected by the chronic NO’ treatment, and that the remaining mitochondria are working at a higher rate to support the energetic demands of the cell. Importantly, all
of these effects were seen with sub-toxic concentrations of the NO$^-$ donor. This indicates that the depletion of the reserve capacity might be involved in the sensitization to cell death also demonstrated in this chapter.

**The Reserve Capacity in vivo**

The concept of a bioenergetic reserve capacity has been present in the literature for some time now, and is typically discussed with reference to exercise endurance and muscle fatigue. These experimental models have examined the difference between the resting oxygen consumption in a whole tissue or an entire organism, and the maximal oxygen consumption in response to intense exercise. The first data demonstrating a quantitative measurement of the reserve capacity was performed using $^{31}$P NMR to measure phosphocreatine levels in skeletal muscle tissue. These experiments indicated that there is a significant ability to increase oxygen consumption to produce high energy phosphate (ATP or phosphocreatine) in response to intense exercise. Importantly, these measurements were performed in whole organisms, or in specific tissue regions (e.g. heart or forearm). This allowed the authors to examine the stimulation of oxygen consumption and uptake by muscle tissue. Muscle tissue is an ideal model for these studies because of the large energy requirements, the ability to rapidly stimulate energy demand, and the utilization of both ATP and phosphocreatine as energy sources. As both of these are usable by muscle for contraction, and visible by $^{31}$P NMR, a comparison to inspired oxygen can be made. Together, these studies demonstrate the presence of a reserve capacity at the organism or tissue level. Furthermore, the importance of this reserve capacity is ap-
parent due to the rapidity with which oxygen consumption can be stimulated in these tissues during exercise.

Beyond the role for a reserve capacity in athletes, the reserve capacity has also been demonstrated to play a role in several diseases. Specifically, failure of the metabolic reserve has been implicated in heart failure [45, 156], and diabetes [276, 277]. These diseases demonstrate a need for “metabolic remodeling” [45]. In the volume overloaded heart, the heart becomes physically larger to compensate for the increased pre-load placed on the organ [278, 279]. Concurrent with this physical remodeling, and eventual failure, mitochondrial metabolism is unable to maintain ATP generation to cope with the increased demand [280]. The failing heart has been reported to have up to a 30% decrease in ATP levels as compared to normal hearts [281]. This resulting disparity between supply and demand for ATP has been suggested to play an important role in the progression of heart failure [45, 156, 160, 281]. Thus, the study of mitochondrial bioenergetics in models relevant to cardiovascular diseases is of increased importance.

Mitochondrial Disease and the Reserve Capacity

The penetrance of mitochondrial diseases appears to be very low due to a similar “reserve capacity” concept. Typically, diseases associated with mitochondrial DNA mutations are rarely seen to have widespread effects on whole body energetics. Tissues affected typically are those with a large demand for energy production from the mitochondria, and are usually neuronal, or motor-related. This is thought to be due to the presence of a large reserve capacity for ATP production in cells. Thus there is a requirement for
an approximate 70% defect in the respiratory chain activity before any phenotype is present. The effects of these diseases are often seen in muscle and neuron due to the high energy demands in these tissue types. Muscle fibers require large amounts of high energy phosphate in the form of ATP and phosphocreatine to enable contraction of the myosin apparatus. Similarly, neurons transport large amounts of ions across the plasma membrane, often through the use of ATP-dependent ion pumps. This occurs with high frequency during action potential firing, thus creating a need for large amounts of energy production. Thus, these tissues are more likely to demonstrate a mitochondrial disease phenotype when defects or deletions of the mtDNA are present. Similar to the discussion of the importance of the reserve capacity in heart failure above [45], it is likely that these mitochondrial diseases manifest in high energy tissues due to the depletion of the cellular reserve capacity. It is thus interesting to speculate that stimulation of the bioenergetic function by increasing the reserve capacity may offer a therapeutic target in patients with diseases of mitochondrial metabolism. This concept will be discussed in further depth at the end of this chapter.

Summary

In summary, the data presented in this thesis clearly indicate the presence of a reserve capacity in intact cells. For the first time we have quantified this parameter, and more importantly have measured its loss in response to stressors relevant to cardiovascular disease. These data suggest that the reserve capacity is an important modulator of cell survival in response to acute stress. Additionally we show that chronic stressors, such as long-term NO` treatment decrease the reserve capacity, sensitizing cells to secondary ox-
idant stress. This may play a role in controlling endothelial cell function in the development of atherosclerosis, and in the response of myocytes to ischemic insult. The concept of a reserve capacity modulating cellular responses is shown schematically in Figure 6-6. In this figure, the reserve capacity is shown to protect against cellular stressors, thereby extending the point at which toxicity starts. Depletion of the reserve capacity as discussed above would likely shift the concentration curve leftward, and thus lower concentrations of stressor would cause overt cell death. Similarly, increasing this reserve capacity potentially shifts the curve rightward. This would extend the protection afforded by the reserve capacity, and allow for resistance to higher concentrations or times of exposure to stressors. Modulation of the reserve capacity will be discussed in further depth at the end of this chapter.

**PRECONDITIONING AND CELLULAR BIOENERGETICS**

**Preconditioning in Elite Athletes: Towards Bioenergetic Therapeutics**

As discussed in previous chapters and above, the data presented in this thesis support the idea that the reserve capacity plays a central role in the cellular response to stressors. It is thus interesting to postulate that this reserve capacity could potentially be modulated to therapeutic benefit. As with the existence of the reserve capacity, the concept of stimulating this respiratory parameter has also been suggested previously. Athletes have long referenced the lactate threshold as a sort of reserve capacity. The lactate threshold is defined as the point at which blood lactate levels begin to accumulate during intense exercise due to production outpacing breakdown. As the body’s immediate energy stores are depleted from the muscle tissue, cells turn to anaerobic metabolism to sus-
Figure 6-6: The importance of the reserve capacity in controlling cell fate. The data discussed in this thesis suggest that a critical determinant controlling cell death in response to various stressors is the reserve capacity. These data demonstrate 3 distinct methods of reserve capacity utilization, and that exceeding the reserve capacity can lead to cytotoxicity. This scheme demonstrates this concept. Under normal conditions, or at times when the cell is responding to low-level stress, the reserve capacity allows for increased $O_2$ consumption, and protects from cell death. As the strength or duration of the stressor increases, the reserve capacity is depleted, and eventually fails. This now allows for cytotoxic pathways to be initiated and leads to overt cell death.
tain ATP levels. During anaerobic metabolism, there is a large utilization of glucose which is accompanied by an increase in lactate production. This lactate accumulation results in the painful burning sensation that occurs during exercise. Furthermore, polymorphic alleles which are positively associated with increased respiratory reserve and higher maximal oxygen consumption have been shown to correlate with elite athlete status [282]. Similarly, intensive muscle training in rats [283] and humans [284] has been shown to increase oxidative capacity. These findings suggest that repeated exercise leads to a preconditioning whereby the reserve capacity is increased. One proposed mechanism of this effect is through the stimulation of mitochondrial biogenesis [285]. Importantly, this preconditioning could be protective when the reserve capacity must be used to cope with disease.

**Can the Reserve Capacity be Increased?**

The data discussed in this thesis thus raise one major question with regard to the future of study in this field: Can the reserve capacity be increased? As discussed above, we have found several mechanisms whereby the reserve capacity is decreased. Only in a few small instances presented in this chapter is the total bioenergetic capacity of the cell, including the reserve capacity, increased. Importantly, the concept of a stimulable reserve capacity has been accepted for some time now. Athletes train and condition prior to events in order to be able to run longer, swim farther, cycle faster, and generally have increased endurance during the event. This is conceptually related to the stimulation of the reserve capacity within one cell. The current view of cellular bioenergetics holds that the reserve capacity exists to allow for stimulated energy production during times of in-
creased need. The data presented in this thesis support this concept by demonstrating utilization of the reserve capacity in response to various cellular stresses.

Figure 6-7 shows a small sample of potential modulators of the reserve capacity. Generally these can be divided into four main groups. Proliferative agents and stimulators of mitochondrial biogenesis are thought to positively affect the reserve capacity. Conversely, cytostatic agents and ATP-consuming processes either inhibit the reserve capacity, or utilize the existing reserve capacity to decrease the remaining reserve capacity. These pathways are thus those that could lead to therapeutic interventions to modulate the bioenergetic potential of a cell system, or a person. Notably, this modulation could be to either increase or decrease the bioenergetic reserve. Drugs designed to target these pathways are already in wide use. Specifically, cytostatic agents have been among the first-line options for anti-cancer therapy for over 20 years, and they have been of interest for much longer [286]. Similarly, PPARγ agonists have been in use for the treatment of diabetics for over a decade, and are quickly becoming one of the first-line agents targeting insulin resistance in patients with Type 2 Diabetes Mellitus. Similarly, the beneficial effect of resveratrol, a chemical found in grape skins, has been largely attributed to its ability to activate SIRT1. Indeed, the most intriguing target for stimulation of the bioenergetic reserve capacity is the AMPK-SIRT1-PGC-1α signaling axis.

Silent Information Regulator T1 (SIRT 1) is a deacetylase that is dependent on NAD⁺ for its activity. This reliance on NAD⁺ allows for precise modulation by the cellular metabolic status. As cellular NAD⁺ increases, SIRT1 can activate many targets, in-
Figure 6-7: Potential modulators of the reserve capacity. Many physiological, pathological, and therapeutic processes are thought to impact on the cellular reserve capacity. Broadly, these could include proliferative agents and stimulators of mitochondrial biogenesis which could increase the mitochondrial mass, and thus the availability of ATP-generating machinery. Negative modulators of the reserve capacity could be cytostatic agents, or processes that stimulate the consumption of ATP. These processes would thus lead to a utilization of the reserve capacity, and thus a decrease in the available reserve capacity. The heterogeneity of the pathways and processes shown here points towards a central role for the reserve capacity in controlling cell fate in response to bioenergetic stressors.
cluding Peroxisome Proliferator-Activated Receptor γ-Coactivator 1α (PGC-1α). Similarly, AMP-Activated Protein Kinase (AMPK) is sensitive to the cellular AMP/ATP ratio. As the AMP levels rise, indicating a low energy state, AMPK becomes activated. Interestingly, AMPK is also known to activate PGC-1α. Thus the activation of PGC-1α is largely controlled by two pathways that are sensitive to the cellular metabolic state [287]. Importantly, activation of PGC-1α has been shown to increase mitochondrial biogenesis, fatty acid oxidation, insulin sensitivity, and mitochondrial oxygen consumption [288, 289]. SIRT1 also increases fatty acid oxidation [290] and gluconeogenesis [291] and decreases NF-κB activation [292]. Furthermore, cross-talk between these two energy-sensing pathways has been suggested, allowing for exquisite control over cellular bioenergetics [293]. Stimulation of this pathway from any of a multitude of directions could then provide for positive therapeutic benefit in the increased bioenergetic capacity. Indeed the SIRT pathway has been suggested as a potential target for drug development [294]. Stimulation of SIRT1 has already been shown to offer protection in cell culture models of diabetes [295] and was protective in a transgenic mouse model fed a high-fat diet [296]. Curiously, a polyphenol found in red wine called resveratrol has been demonstrated to activate SIRT1 in a number of models [297]. Activation by resveratrol has been suggested to protect from oxidative stress [298], improve mitochondrial function [299], decrease markers of aging [300] and extend life span [301]. Additionally, resveratrol has been studied for improving energy homeostasis in neurons [302, 303] and in mouse models of Type 2 diabetes [304]. Together, this large body of work indicates that the AMPK-SIRT1-PGC-1α pathway may be an important modulator of the cellular bioenergetic capacity, and may directly impact on the reserve capacity. Importantly, the
study of these pathways as they relate to cellular energetics has only begun in earnest in the last decade. Much is not understood in the regulation of this pathway and the true therapeutic potential in preventing disease pathogenesis. Future studies in our lab will investigate the involvement of this pathway in the modulation of the reserve capacity, and will serve as a starting point for examining mechanisms of reserve capacity stimulation.

CONCLUSIONS

As mentioned in Chapter 1, a great deal is known regarding the changes in mitochondrial oxygen consumption during the progression of many diseases. Most of the studies reported utilized animal model systems to examine mitochondrial function in isolated tissue samples. Probing mechanisms of pathogenesis related to mitochondrial dysfunction has thus been difficult because of the requirement for large tissue samples and lack of a cellular context once mitochondria are isolated. The data in this thesis demonstrate the use of a novel technology to determine and quantify mitochondrial function in cell culture systems. This has allowed for an approach to mechanisms of mitochondrial dysfunction in response to various stimuli. The methods for determining changes in mitochondrial function were discussed in detail in Chapter 2. The mitochondrial function assay described in that chapter allowed for in depth analysis of the response to other cellular stressors. In Chapter 3, acute treatment with nitric oxide was shown to decrease the reserve capacity, but not to impact on the basal oxygen consumption. Data in Chapter 4 demonstrated for the first time a stimulation of oxygen consumption in response to HNE. Previous studies only demonstrated a decline in mitochondrial State 3 respiration in response to this compound, possibly due to the lack of a cellular context. As shown in
Chapter 5, chronic treatment with nitric oxide decreased the cellular reserve capacity; however, this occurred through a mechanism distinct from the data discussed in the other two chapters. Collectively, the data discussed herein represent a new view of the role of mitochondrial function in response to cellular stress. Importantly, we have defined a new role for the reserve capacity in the protection from these stressors. Data in Chapters 3, 4, and 5 all show that depletion of the reserve capacity correlates with increased cytotoxicity and susceptibility to secondary stressors. This has led to the concept of a threshold that controls the cellular response to stressors. The data presented here suggest that only when this threshold is overextended is there overt cell death. Importantly, these concepts extend our understanding of the role of this reserve capacity in the cellular response to stressors.
REFERENCES


