Targeting the mitochondrial inner membrane to improve bioenergetics in the diseased heart

by

Fatiha Moukdar

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Director of Dissertation: David A. Brown

Major Department: Physiology

Cardiovascular diseases continue to exact unparalleled economic and humanitarian costs across the globe. Manifestations of cardiovascular diseases include acute coronary syndromes and heart failure, both of which are exacerbated in diabetic patients. Although the underlying cellular culprits responsible for these cardiomyopathies are multi-factorial, aberrant cellular bioenergetics is emerging as a central component. Decrements in mitochondrial function impair cardiac function, and accordingly the development of novel therapies that improve cardiac function by targeting mitochondria has enormous therapeutic potential. In the work presented herein, we studied two diseases where impaired bioenergetics comprises a central component: diabetes, and ischemia/reperfusion injury. In diabetic heart studies, we determined the mechanisms responsible for the decline in mitochondrial bioenergetics of the diabetic heart. Comprehensive mitochondrial functional assays coupled with molecular techniques were employed. Our results showed that mitochondrial respiration and reactive oxygen species buffering capacity were significantly decreased in diabetic hearts. Diabetic mitochondria displayed aberrant mitochondrial calcium handling, post-translational oxidative modification of the adenine nucleotide translocase, increased sensitivity to permeability transition pore opening,

and lowered overall expression of proteins involved in the electron transport system. These effects led to inefficient energy supply-demand matching and heightened reperfusion injury in intact heart studies. Treatment with several novel therapies that target the mitochondrial inner membrane reduced the extent of injury and restored mitochondrial function in the diabetic heart. In ischemia/reperfusion studies, we tested the hypothesis that aberrant respiration in the post-ischemic heart was due to impaired molecular organization along the inner mitochondrial membrane. Specifically, we used a novel respiratory substrate-inhibitor titration protocol to determine complex-specific changes, in the electron transport system, that lead to poor respiration. These respiratory studies were coupled with experiments using native gel electrophoresis, allowing us to link changes in respiration to altered expression of native respiratory "supercomplex" clusters. The decrease in mitochondrial respiration after ischemia/reperfusion was observed along several different sites of the electron transport system. These changes were associated with lower supercomplex expression, and altered levels of several native respiratory complexes. Post-ischemic treatment with a mitochondria-targeting peptide restored supercomplex assembly and was associated with improved respiration and a decreased extent of injury. Taken together, the results presented herein provide new insight into the molecular and functional alterations that occur along the mitochondrial inner membrane in diabetic and post-ischemic hearts. These data provide a basis for novel therapies targeting the inner mitochondrial membrane as viable pharmacological approaches to improving bioenergetics in diseased myocardium.

Targeting the mitochondrial inner membrane to improve bioenergetics in the diseased heart

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APPROVED BY:

DISSERTATION ADVISOR: _____
David A. Brown, Ph.D.

COMMITTEE MEMBER:
_____
David A Tulis, Ph.D.

COMMITTEE MEMBER:
_____
P. Darrell Neufer, Ph.D.

COMMITTEE MEMBER:
_____
Robert M. Lust, Ph.D.

COMMITTEE MEMBER:
_____
Saame Raza Shaikh, Ph.D.

CHAIR OF THE DEPARTMENT OF PHYSIOLOGY
_____
Robert M. Lust, Ph.D.

DEAN OF THE GRADUATE SCHOOL
_____
Paul J. Gemperline, Ph.D.

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+ dP/dt	Maximal rate of contraction
-dP/dt	Maximal rate of relaxation
ADP	Adenosine diphosphate
ANT	Adenine nucleotide translocase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUR	Amplex Ultra Red Reagent
BCA	Bicinchoninic acid
Bpm	Beats per minute
BN-PAGE	Blue native polyacrylamid gel electrophoresis
BSA	Bovine serum albumin
C	Complex
Ca	Calcium
CsA	Cyclosporine A
CHD	Coronary heart disease
CVD	Cardiovascular disease
CyP-D	Cyclophilin D
DDM	n-dodecyl-β-D-maltoside
DTT	Dithiothreitol
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
Em	Emission
ETC	Electron transport chain
Ex	Excitation
FCCP	Carbonyl cyanide p-trifluoromethoxy phenylhydrazone
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

i.p.	Intraperitoneal
IRI	Ischemia-reperfusion injury
IS	Isolation solution
LV	Left ventricle
LVDP	Left ventricular developed pressure
MCU	Mitochondrial calcium uniporter
MCUR1	Mitochondrial Ca uptake regulator 1
MICU1	Mitochondrial Ca uptake 1
MRC	Mitochondrial respiratory complexe
MVO_2	Myocardial oxygen consumption
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NEFFA	Non-esterified free fatty acids
Pi	Inorganic phosphate
PDH	Pyruvate dehydrogenase
PTP	Permeability transition pore
R	Gaz constant
RCR	Respiratory control ratio
ROS	Reactive oxygen species
SERCA	Sarco/endoplasmic reticulum Ca ATP-ase
SH	Thiol group
SEM	Standard error of the mean
STZ	Streptozotocin
T	Temperature
TBS-T	Tris-buffered saline containing 0.1% Tween
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
TPP+	Tetraphenylphosphonium ion
TTC	Triphenyltetrazolium chloride
v	Mitochondrial matrix volume
V	Buffer volume
VDAC	Voltage dependent anion channel

ΔE	Deflection in TPP+ voltage from baseline
ΔΨm	Mitochondrial membrane potential

Chapter 1: Introduction

Cardiovascular disease

In the United States, the overall rate of death that was attributed to cardiovascular disease (CVD) in 2010 reached 235.5 per 100000 [1]. On this basis, approximately 2150 Americans die of CVD each day, averaging 1 death every 40 seconds. Coronary heart disease (CHD) alone caused about 1 of every 6 deaths reaching a total of 379559 deaths in 2010 [1].

Each year, an estimated 620000 Americans have a new coronary attack and around 295000 have a recurrent attack. Furthermore, it is estimated that an additional 150000 silent first myocardial infarctions occur each year. To put these statistics together, approximately every 34 seconds 1 American has a coronary event, and approximately every 1 minute 23 seconds an American will die of one [1].

In addition to costing lives, CVD has a big economical impact as well. Indeed, the total direct and indirect cost of CVD and stroke reported for 2010 was estimated to be $315.4 billion [1]. Therapeutic strategies that diminish the burdens of this disease are a matter of utmost importance.

In order to have an efficient therapeutic approach, key cellular components responsible for initiation/complications of CVD need to be identified and targeted. Using IRI and diabetes as experimental models, we addressed the structural and molecular changes of critical mitochondrial proteins, the impact of these changes on mitochondrial function, and ultimately on the cardiac function. We specifically assessed the link between mitochondrial defects and the myocardial infarct in diabetic and non-diabetic hearts, as well as mitochondrial defects and energy-supply demand mismatching in diabetic hearts. Lastly, we evaluated the beneficial effect of Bendavia in each of our experimental models. Bendavia (also called SS-31 or MTP-131) is a

cell-permeable tetra-peptide that targets mitochondria [2]. This peptide has been shown to preserve mitochondrial function, to reduce oxidative damage and to protect against cellular death following ischemia/reperfusion injury (reviewed in [3]). This peptide is currently being tested in the EMBRACE-STEMI clinical trial for acute coronary syndromes [4].

Ischemic heart disease

The American Heart Association defines CHD as the result of plaque buildup in the coronary arteries. Overtime, this plaque can chronically limit blood flow to areas of the myocardium by narrowing the coronary artery or it can acutely rupture causing a heart attack. Areas of myocardium that have limited blood flow, as a result, are said to be ischemic. While re-establishing blood flow to these areas is essential, it has also been reported to be damaging. The damage to the myocardium associated with an ischemia/reperfusion event is referred to as IRI, and is identified as the primary cause of sudden cardiac death [5-7].

During ischemia, oxidative phosphorylation is diminished due to the fall in oxygen levels, and this leads to a significant and quick decline in cardiac ATP content. To compensate for this energy loss, the myocardium switches to anaerobic metabolism; however, this switch leads to a cytosolic acidification. The low pH and aberrant ATP-dependent pump/exchanger activities results in a net accumulation of Ca inside the cardiac cell (reviewed in [8]).

Upon reperfusion, two main events take place leading to the opening of mitochondrial PTP and subsequent cellular death. These two events are the high levels of ROS generated following the sudden burst in oxygen at reperfusion and the mitochondrial Ca overload that takes place following mitochondria repolarization [9-11].

Interventions that aim at preventing Ca overload by blocking the mitochondrial Ca uniporter, along with those that decrease oxidative stress have been shown to be protective

against IRI (reviewed in [12]). Results reported in this dissertation (chapters 2 and 3) contribute to advancing our knowledge on the regulation of specific mitochondrial proteins involved in mitochondria Ca handling and PTP opening. More importantly, these results shed the light on the regulation of these proteins in the context of IRI and diabetes and illustrate the clinical potential of the mitochondria-targeted peptide Bendavia.

Ischemic heart disease and diabetes

Diabetes affects 25.8 million Americans (8.3% of the U.S. population) [13] and 346 million people worldwide [14]. It is a major cause of heart disease and stroke. In fact, death rates from heart disease and the risk of stroke are about 2 to 4 times higher among adults with diabetes than among those without diabetes [13]. This increased cardiac mortality rate is independent of both coronary artery disease and hypertension [15], suggesting an underlying defect at the level of the cardiomyocyte [16].

Among the myriad factors involved in the increased cardiac mortality rate in diabetic patients, recent evidence suggests that decrements in bioenergetics coordination are centrally involved. While the ***healthy*** heart is proficient at adjusting its mitochondrial ATP production to changing workloads, the diabetic heart has a very limited capacity of energy matching [17, 18]. A growing body of literature indicates that alterations in mitochondrial function may be responsible for the increased cardiac dysfunction observed in diabetes (reviewed in [19]).

Diabetic hearts also exhibit oxidative stress characterized by an increase in ROS production [20, 21] and a decreased capacity to buffer them [22-24]. We believe that a direct consequence of this oxidative shift in cellular redox environment is an oxidative modification of key mitochondrial protein complexes that eventually lead to either their degradation or dysfunction.

Calcium plays a key role in both the optimal function and dysfunction of the mitochondria. Physiological levels of mitochondrial Ca are vital for ATP generation which is crucial for cardiac function, but Ca overload leads to mitochondrial dysfunction and cellular death. For this reasons, mitochondrial Ca uptake and the nature/identity of the mitochondrial Ca uniporter have been the subject of multiple investigations over the last 50 years. Understanding how mitochondrial Ca uptake is regulated in health and disease can help us better understand the contribution of mitochondrial Ca to the cardiac energy supply-demand matching under physiological and pathological conditions.

To address the changes that might impact mitochondrial Ca uptake in the diabetic heart, we looked at the molecular alterations of mitochondria in healthy and diabetic hearts. More specifically, we measured the levels of mitochondrial Ca handling proteins and evaluated the link between these levels and the overall mitochondrial function (chapter 3). The goal was to advance our understanding of the mechanisms responsible for the known impaired cardiac mitochondrial Ca dynamics in diabetes. We accomplished that by identifying and measuring the molecular changes of key proteins that are directly or indirectly involved in ATP generation (chapter 3).

The role of mitochondria in cardiac function

The main function of mitochondria is ATP synthesis *via* oxidative phosphorylation. After substrate oxidation, reducing equivalents generated by the tricarboxylic acid cycle are used by the electron transport chain (ETC) to generate the mitochondrial membrane potential that drives ATP synthesis [25]. For the heart, this ATP synthesis is crucial to its main function which is to continuously supply the whole body with oxygen and nutrients. This task requires the heart to match its energy supply to demand on a beat-to-beat basis, and this is reflected by the important mass of cardiac mitochondria, as it occupies 35% of the cardiac cell volume [26]. As an example of the constant energy matching, a 7-fold increase in cardiac output results in a 10-fold increase

in myocardial oxygen consumption. The latter is a direct index of ATP consumption by the heart [27]. So, constantly matching cardiac ATP supply to demand is critical to ensure sufficient fuel availability for the heart to perform its function. Therefore, a constant communication between the cytosol (compartment of energy consumption) and the mitochondria (compartment of energy generation) is essential.

Two mechanisms have been proposed as possible regulators of this communication between the cytosol and mitochondria and the ultimate regulation of ATP synthesis. First, the levels of adenosine di-phosphate (ADP) and inorganic phosphate (Pi) because an increase in ATP utilization in the cytosol leads to an increase in ADP and Pi fluxes to mitochondria, and a concomitant increase in the amount of available substrates for ATP production. Second, free cytosolic Ca concentration because it is coupled with 1) ATP utilization by the contracting cardiac muscle, 2) mitochondrial Ca fluxes, and 3) Ca-dependant activation of mitochondrial enzymes involved in ATP production (reviewed in [28]). In the work presented here we looked at the molecular control of mitochondrial Ca fluxes in relation to cardiac bioenergetics (chapter 3).

A new paradigm for optimal mitochondrial function has recently emerged. It is the structural organization of the mitochondrial respiratory complexes (MRCs) into supramolecular configuration called supercomplexes. When the MRCs are organized as such, electron transfer through partner redox components is more efficient [29], and electron loss that leads to ROS formation is minimal [30]. This electron transfer efficiency might result in an optimal energy production, especially that the organization of these supercomplexes is said to be dependent and regulated by the type of substrate used [31].

The mitochondrial respiratory complexes

The ETC is composed of five multi-subunit complexes: NADH-ubiquinone oxidoreductase (commonly known as complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome c oxidoreductase (cytochrome bc1 complex or complex III), cytochrome c-O_2 oxidoreductase (complex IV), and F1Fo-ATP synthase (complex V), in addition to 2 mobile electron carriers, ubiquinone and cytochrome c [25]. Complexes I and II are the two major routes of entry of electrons into the ETC. The electrons are then transferred through a series of redox centers in complex III and IV until reaching the molecular oxygen which is the last electron acceptor. Complexes I, III and IV couple electron transfer with the pumping of protons from the mitochondrial matrix into the intermembrane space thus, generating a proton gradient across the inner mitochondrial membrane. Complex V utilizes the potential energy stored in this proton gradient to regenerate ATP [32].

Structural organization of the mitochondrial respiratory complexes

The structural and functional organization of the MRCs has been subject of multiples investigations since they were successfully purified from bovine heart mitochondria in 1962 [33]. Currently, there is evidence for 2 models describing the organization of MRCs; the fluid-state model and the solid-state model.

Fluid-state model

In this model, the respiratory complexes are described as free units that are randomly dispersed and able to move by lateral diffusion through the lipid bilayer [34]. Electron transfer is facilitated by the movement of ubiquinone, transferring electrons from complex I/II to complex III, and cytochrome c, transferring electrons from complex III to IV. This model is the most described in the literature and is widely accepted.

Solid-state model

The observation that the MRCs are not present in a stoichiometry of one-to-one, brought Hochman et al. to propose a "dynamic aggregate model" in which the components of the ETC exist as complete aggregates, partial aggregates and freely diffusing components, all active in electron transfer [35]. In this model, electron transfer can occur by random diffusion, but higher rates are achieved by formation of transitory complexes between appropriate redox partners.

This dynamic aggregate model hypothesis was the first statement in favor of the existence of a supramolecular organization of the MRCs. Hochman et al. described the function of these dynamic aggregates as follows: *"In so far as such associated forms exist, the efficiency of electron transfer will increase and overall rates will be observed that are faster than those predicted by a totally random diffusion process. A corollary of the hypothesis is that changes in the stability or lifetime of aggregated forms may provide a mechanism of regulation, in addition to normal respiratory control*" [35].

Today, evidence based on the separation of MRCs by blue native-polyacrylamide gel electrophoresis (BN-PAGE), suggests that the respiratory complexes I, III, and IV can associate in different stoichiometries to form supramolecular assemblies with functional features, called supercomplexes or respirasomes [31, 36-38]. As illustrated in Figure 1, these organizations promote optimal vicinity between different complexes and result in a more efficient electron transfer from one complex to the other thus, improving mitochondrial function [29] and decreasing electron loss and ROS generation [30, 31]. Besides the BN-PAGE analysis, supercomplexes structure was also confirmed by electron microscopy analysis [39], cryoelectron microscopy [40], and single particle analysis [40, 41].

Cardiolipin and the mitochondrial function

Cardiolipin is a phospholipid found mostly in the inner mitochondrial membrane where it represents around 30% of the total lipid content (reviewed in [42]). This phospholipid is essential for the optimal structure, stability and activity of multiple MRCs including comlexes I and V (reviewed in [42]). Cardiolipin is also critical for the organization of MRCs into respirasomes since it helps hold the different complexes together [43]. Cardiolipin peroxidation and/or loss were reported and linked to mitochondrial dysfunction under different disease states including ischemia/reperfusion injury and heart failure (reviewed in [42]). Recent evidence shows that Bendavia might exert its protective action by targeting cardiolipin [44].

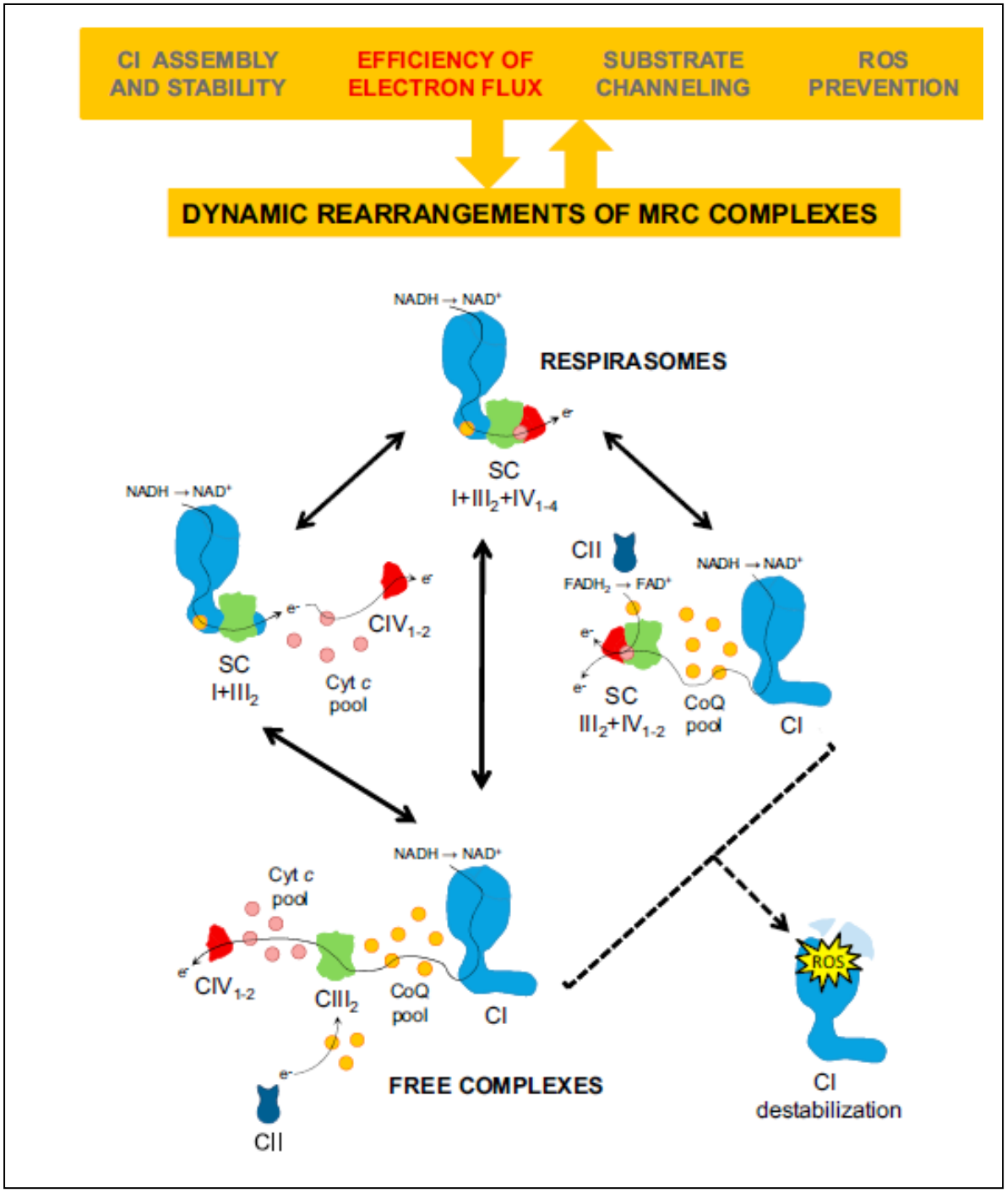


Figure 1: Organization of the mitochondrial respiratory complexes into free complexes or supercomplexes (respirasomes). From [45] with permission (see Appendix B). Respirasomes are formed by the assembly of respiratory complexes I, III and IV in different stoichiometries.

Calcium and cardiac bioenergetics

The heart is capable of sustaining adequate cardiac function with changing metabolic demand by continuously balancing the rate of mitochondrial ATP production with utilization. Ca initiates and powers muscle contraction which makes it an ideal feed-forward signal for priming ATP production. In fact, Ca plays a key role in the cardiac constant phosphorylation potential because it signals the cytosolic needs in ATP to the energy generating machinery of the mitochondria [46, 47].

For a long time mitochondria was considered to play a role in Ca buffering during diastole; however, it is now clear that mitochondrial Ca uptake is essential to matching energy supply to demand. Once inside the mitochondrial matrix, Ca actively stimulate several dehydrogenases of the Krebs Cycle as well as ANT, and the F1/Fo ATP-synthase [46-51]. The activity of these proteins is essential for ATP synthesis and delivery to the contractile machinery.

Mitochondrial Ca influx occurs primarily *via* the mitochondrial Ca uniporter (MCU), an ion channel within the inner mitochondrial membrane whose molecular identity has recently been elucidated [52, 53]. The activity of MCU is regulated by at least 2 newly characterized mitochondrial proteins: Mitochondrial Ca uptake 1 (MICU1) [54] and mitochondrial Ca uptake regulator 1 (MCUR1) [55], and its physiological role in mitochondrial Ca uptake, as well as the consequent energy supply-demand matching has recently been further confirmed [56]. By using a transgenic mouse lacking MCU, Pan and Colleagues showed that in the absence of MCU, the animals lose the ability to rapidly uptake Ca, and exhibit alterations in the phosphorylating activity of pyruvate dehydrogenase [56].

While it is well known that mitochondrial Ca influx is decreased in the diabetic heart [57-59], there are no data linking this decrease to changes in activity/expression of Ca-handling

proteins/channels. Further, whether these proteins can be regulated by a therapeutic intervention is not known. In chapter 3, we address those questions in diabetic and non-diabetic hearts.

Conclusion and central hypothesis

Heart disease is the leading cause of death for both men and women [60] and several medical conditions can put individuals at higher risk for this disease. Diabetes is a major risk factor as well as a complicating condition for heart disease. Indeed, diabetic patients are more vulnerable to this disease, and have lower rate of recovery from myocardial ischemic events. The underlying pathological factors of the diabetic state, such as the high oxidative burden, the poor handling of Ca, and the energetic inefficiency all contribute to the increased susceptibility of diabetic patients to IRI. Multiple investigations have identified mitochondria as the culprit in both IRI and diabetes, and proposed some therapeutic approaches that would reverse/diminish the mitochondrial dysfunction. However, we are still awaiting drug/drugs that will have an application in the clinic, will reach the mitochondrial target/targets, and improve the recovery of cardiac function after an ischemic event. The fact that therapies such as antioxidants have not been successful in the clinical setting [61] is an indicator of our limited understanding of all the molecular and structural changes that take place in the mitochondria under diabetic and ischemic conditions. The working premise of this dissertation is that diabetes and IRI overwhelm the intrinsic mitochondrial antioxidant capacity, and result in an oxidative shift of the cellular redox state. This shift can lead to oxidative modifications of key mitochondrial proteins as well as the environment surrounding these proteins, and result in declined mitochondrial bioenergetics. We propose to identify the molecular and structural modifications taking place at the level of the inner mitochondrial membrane, under two diseased states: IRI and diabetes. We hypothesize that: 1) the increased susceptibility of diabetic hearts to IRI is due to an increased sensitivity to PTP opening, 2) the cardiac inefficiency of diabetic hearts is, at least in part, due to

alterations of mitochondrial Ca handling proteins as well as an overall declined mitochondrial function and 3) the mitochondrial dysfunction in the post-ischemic heart is due to impaired molecular organization along the inner mitochondrial membrane.

By using a mitochondria-targeting peptide that has been shown to be protective against mitochondrial dysfunction in different cardiac diseases, we hope to pinpoint its main mitochondrial target/targets and to better understand the changes that take place in the mitochondria of diabetic and ischemic hearts.

Chapter 2: Mitochondrial permeability transition in the diabetic heart: Contributions of thiol redox state and mitochondrial calcium to augmented reperfusion injury.

From: Ruben C. Sloan, Fatiha Moukdar, Chad R. Frasier, Hetal D. Patel, Phillip A. Bostian, Robert M. Lust, David A. Brown. Journal of Molecular and Cellular Cardiology, 52 (2012): 1009–1018 (http://dx.doi.org/10.1016/j.yjmcc.2012.02.009) [62]

Introduction

The global population of diabetics is estimated to reach 300 million by the year 2025 [63]. Among the myriad complications associated with diabetes mellitus, diabetics have a higher mortality rate following myocardial infarction [64], with hyperglycemia independently increasing the mortality risk [65]. Animal models of diabetes indicate increased ischemia/reperfusion injury in diabetic hearts [66-68], but the underlying mechanisms responsible for augmented injury in diabetic hearts are not fully understood. Insight into the cellular pathways involved in diabetic ischemic injury has potential to foster novel therapeutic strategies seeking to diminish ischemic injury in the rapidly growing diabetic population.

While the etiology of ischemia/reperfusion injury is undoubtedly multi-factorial [8], a growing body of evidence suggests that opening of the mitochondrial permeability transition pore (PTP) is involved (reviewed in [19]). Diabetic heart mitochondria demonstrate an enhanced susceptibility to PTP opening in both humans [66] and animal models of diabetes [69-71], but the mechanisms contributing to augmented PTP opening in diabetes are not known. Studies in non diabetic animals have demonstrated that PTP opening is favored when mitochondrial levels of ROS and calcium are high [9, 72-75]. Increased mitochondrial ROS production ultimately leads to oxidation of protein thiols associated with the PTP, which is known to increase PTP calcium sensitivity [76].

Given that diabetic heart mitochondria exhibit impaired ROS scavenging [22, 23, 77], particularly by the glutathione redox couple [68, 78, 79], we hypothesized that the enhanced PTP opening in diabetic mitochondria is secondary to oxidative shifts in the cellular redox environment. We hypothesized that modifying the thiol redox state in isolated mitochondria with the thiol reductant dithiothreitol would delay PTP opening in diabetic mitochondria. Further, we speculated that reducing the oxidant burden in vivo with a novel mitochondria targeted peptide (MTP-131, an analog of SS-02 and SS-31; [3, 80]) would also attenuate the PTP open probability in diabetic heart mitochondria. This cell-permeable peptide has been shown to decrease intracellular ROS levels and reduce mitochondrial depolarization in a number of cell types undergoing oxidative stress [80].

To determine if these approaches in mitochondria translate to tissue salvage in the intact hearts, we hypothesized that three different pharmacological approaches administered at the onset of reperfusion would protect tissue by keeping PTP closed. We used NIM811, a non-immunosuppressive analog of cyclosporin-A to block the PTP [81], minocycline to block mitochondrial calcium influx [82], and MTP-131 to reduce mitochondrial ROS production during reperfusion.

Methods

Experimental animals

All animal procedures received prior approval by the East Carolina University Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (7–9 weeks old) were housed in a temperature (22°C) and light-controlled (12 hour light/12 hour dark) environment and fed standard rat chow (Research Diets, New Brunswick, NJ, USA) and water ad libitum.

After at least 5 days of acclimation to the facility, diabetes was induced with a single intraperitoneal (*i.p.*) injection of streptozotocin (STZ, 65 mg/kg) dissolved in 100 mM sodium citrate (pH=4.5) following a 12-hour overnight fast. Control animals received an i.p. injection of sodium citrate. All experiments were performed 2 weeks following STZ injection. Blood glucose was determined from tail vein samples using a commercially available glucometer (One Touch Ultra 2, LifeScan, Milpitas, CA, USA). Plasma non-esterified fatty acids were measured using a commercial kit (#SFA-1, Zen-Bio, RTP, NC), based on the method described by Johnson and Peters [83] and following the manufacturer's instructions.

On the day of experimentation, rats were anesthetized with a ketamine/xylazine cocktail (90 mg/kg and 10 mg/kg, respectively; i.p. injection) and hearts were removed via bilateral thoracotomy. Once excised, hearts were used in one of three major study arms: 1. Left ventricular glutathione content and immunoprecipitation/western blot experiments for ANT redox state, 2. Experiments in isolated mitochondria, or 3. Ex vivo Ischemia/Reperfusion.

Myocardial glutathione

Left ventricles from a subset of control and diabetic animals (n=5 rats in each group) were excised and frozen for subsequent assays to determine myocardial glutathione content. Total glutathione, oxidized glutathione (GSSG), reduced to oxidized glutathione ratio (GSH/GSSG), glutathione reductase, and glutathione peroxidase were determined using our established protocols [84].

ANT Western blots and thiol redox state assay

Aliquots of LV mitochondria proteins were solubilized, subjected to SDS-PAGE and transferred to an Immobilon-P transfer membrane. After blocking in Tris-buffered saline containing 0.1% Tween (TBS-T) and 4% bovine serum albumin for 1 h at room temperature, the

membrane was incubated with the ANT antibody (1:1000; Santa Cruz) overnight at 4 °C. The membrane was washed with TBS-T and incubated with an IR-Dye-conjugated secondary antibody for 1 h at RT then washed again with TBS-T and finally with TBS. The immunoreactive bands were scanned and quantified using the Odyssey Infrared Imaging system and software, respectively (Li-COR Biosciences, Lincoln, NB).

Free reactive thiol (SH) groups on the adenine nucleotide translocase (ANT) were labeled using IR-Dye 800CW-Maleimide (Li-COR Biosciences) by a modification of the technique described by Riederer et al. [85]. This approach allows maleimide groups to react with sulfhydryl groups of proteins at pH 6.5–7.5 and form a stable thioether bond. Samples of LV were homogenized in buffer (0.1 M Na_2HPO_4, and 0.15 M NaCl, pH 7.5) containing protease inhibitor cocktail and 100 nM IRDye 800CW-Maleimide. The labeled proteins in the supernatant were obtained by centrifugation at 14,000 rpm for 10 min. Excess free IR-Dye 800CW-Maleimide was removed over Zeba columns (Pierce) and protein concentration was determined using BCA kit (Pierce). 500 μg labeled proteins were incubated with 2 μg of the ANT antibody and 40 μL of Protein G PLUS-Agarose beads slurry (Santa Cruz), overnight at 4 °C on a rotating platform. The bound proteins were eluted with Laemmli loading buffer and underwent SDS-PAGE. The gels were scanned and quantified using the Odyssey imaging system and software, respectively.

To validate the effectiveness of the method to detect ANT thiol redox state, 2.5 mg proteins were incubated with either 5 mM H_2O_2 or 5 mM DTT for 30 min at RT on a rotating platform. The excess DTT and H_2O_2 were removed over Zeba columns and the final protein concentration was determined using the BCA kit. The proteins were then subjected to maleimide labeling and underwent SDS-PAGE as described above.

Mitochondrial isolation

Cardiac mitochondria were isolated from the left ventricle of hearts utilizing a protocol similar to Boehm et al. [86]. For the mitochondrial isolation, all steps were performed at 4°C, and all instruments for the procedure were chilled overnight prior to the isolation at 4°C. Hearts were excised and immersed in 10 mL ice-cold isolation solution (IS) containing (in mM): 300 sucrose, 10 sodium-hepes and 0.2 EDTA. The left ventricle was isolated, weighed, and rinsed in fresh IS buffer. Hearts were minced into 2–3 cm^3 cubes and subjected to 2 min of digestion using 1.25 mg trypsin, diluted in 10 mL of IS (pH=7.2). Following digestion, 6.5 mg of trypsin inhibitor was added, diluted in 10 mL IS buffer+BSA (1 mg/mL) at pH=7.4. Tissue was resuspended in 10 mL IS buffer+BSA and homogenized with a teflon Potter homogenizer. The homogenate was centrifuged at 600 g for 10 min, and the supernatant was then centrifuged at 8000 g for 15 min. The supernatant was discarded, and the pellet re-suspended in 10 mL IS buffer+BSA. This step was repeated one more time, and the final pellet was stored on ice in ~150 µL IS buffer. Mitochondrial protein content was determined using a BCA protein assay.

Determination of respiratory control ratio

To determine the quality of our mitochondrial preparations, mitochondrial rates of respiration were measured using a Clark-type micro-oxygen electrode (Microelectrodes, Bedford, NH, USA). Reactions were conducted in a closed, magnetically stirred chamber in 2.5 mL mitochondria assay buffer containing (in mM): 125 KCl, 5 HEPES, 2 KH_2PO_4, 1 $MgCl_2$ and 0.5 mg mitochondria (25°C, pH=7.3). Following a two-minute equilibration period, mitochondria were energized with 5 mM glutamate/5 mM malate to initiate state 2 respiration. State 3 respiration was initiated with the addition of 2.5 mM ADP. Respiratory control ratios were calculated by dividing the state 3 respiration by the state 2 respiration.

Calcium retention capacity

Mitochondria (0.75 mg) were suspended in mitochondria assay buffer (described in section 2.3) and supplemented with the fluorescent probe 1 μM calcium green 5 N salt to track changes in extramitochondrial calcium. Extramitochondrial calcium fluorescence was measured using a fluorescence spectrophotometer (Photon Technology International, Birmingham, NJ, USA), with excitation and emission wavelengths set to 506/532 nm, respectively. Calcium-induced PTP opening experiments were performed under state 2 conditions (5 mM glutamate/5 mM malate). Mitochondrial PTP opening was induced by subjecting mitochondria to serial 50 nmol $CaCl_2$ pulses at 3-minute intervals. PTP induction was denoted by the inability of mitochondria to take up calcium (reflected by a sharp increase in extramitochondrial calcium fluorescence). Calcium retention capacity was quantified as the amount of calcium needed to induce PTP opening (nmol $CaCl_2$/mg mitochondria).

For experiments where redox state was manipulated, energized mitochondria were treated with either 200 μM of the thiol-oxidant diamide or 2 mM of the thiol reductant dithiothreitol (DTT) beginning 10 min prior to beginning calcium pulses. These concentrations of diamide and DTT have previously been used by our group and others [87, 88]. For studies where isolated mitochondria were treated with MTP-131, incubated mitochondria were treated with 1 nM MTP-131 for 10 min prior to beginning calcium pulses.

In vivo MTP-131 treatment

To determine if increased endogenous mitochondrial ROS production was responsible for PTP redox modifications, a subset of rats was treated daily with MTP-131. MTP-131 is a small cell-permeable peptide analogous to SS-02 and SS-31, and is currently being utilized in clinical studies [80]. In this cohort, MTP-131 (1.5 mg/kg in 0.9% saline) was injected (i.p.) daily for the 4 days prior to the mitochondrial isolation day, and calcium retention was determined as described in section 2.6.

Isolated heart studies

For whole-heart studies, rat hearts were perfused (perfusion pressure of 75 mm Hg) in a retrograde fashion on a modified Langendorff apparatus using an established protocol by our group [87, 89, 90]. Hearts were perfused with a modified Krebs–Henseleit buffer containing (in mM): 118 NaCl, 24 $NaHCO_3$, 4.75 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.0 $CaCl_2$, and 10 glucose (gassed with 95/5% O_2/CO_2). Hearts were bathed in a buffer-filled perfusion chamber maintained at 37°C for the duration of the experiments. Following the initiation of perfusion, hearts were instrumented for the simultaneous observation of mechanical and electrical function. A buffer-filled latex balloon (size 5, Harvard Apparatus, Holliston, MA, USA), calibrated at the beginning of each day using a digital manometer, was inserted into the left ventricle (via the mitral valve) for the measurement of left ventricular developed pressure (LVDP), with balloon volume adjusted to establish a diastolic pressure of 5–8 mm Hg. Three electrodes were placed into the buffer-filled perfusion chamber for the measurement of volume-conducted ECG. Coronary flow rates were monitored constantly with a flow probe (Transconic Systems, Ithaca, NY, USA) connected in series with the perfusion line, and normalized to heart wet weight (in grams) at the end of each experiment. All physiological parameters were continuously monitored and stored on a personal computer using commercially available software (Chart, AD Instruments, Colorado Springs, CO, USA). Heart rate was calculated using the LVDP trace, and maximal rates of contraction and relaxation (±dP/dt) were calculated using the derivative of the LVDP trace.

Ischemia/reperfusion protocol and drug treatments

Following a 10 minute baseline period, ischemia/reperfusion was initiated similarly to that described previously by our group [89]. Hearts were exposed to global no-flow ischemia by stopping perfusion for 20 min. At the end of the index ischemia, static buffer from the perfusion

lines was washed out (via an accessory port proximal to the aortic cannula), and reperfusion ensued for 2 h either with control buffer or buffer containing one of three experimental compounds.

Hearts were reperfused with Krebs buffer alone (control), or Krebs buffer containing the PTP blocker NIM811 (5 μM) [82], the mitochondria-targeted peptide MTP-131 (1 nM) [2], the calcium uniporter blocker minocycline (1 μM), or the reducing agent DTT (2 mM). At the end of reperfusion, the left ventricle was dissected, sliced into 5 mm-thick slices, incubated in 1% triphenyltetrazolium chloride (TTC) for 10 min (37°C) and digitally photographed for subsequent infarct size analysis. Infarct size was expressed as a percentage of the left ventricle (calculated using ImageJ software, NIH, Bethesda, MD, USA). Arrhythmias were scored as described previously by our group [87, 89, 90] and were in accordance with the Lambeth Conventions [91, 92].

Materials

Unless otherwise noted, all reagents used were obtained from Sigma-Aldrich. Calcium green 5 N salt was purchased from Invitrogen (Carlsbad, CA, USA). NIM811 was a gift from Novartis, and MTP-131 was obtained from Stealth Peptides Inc.

Statistical analysis

Data are presented as mean±SEM. Statistical analyses were performed using an ANOVA with Newman–Keuls post-hoc analysis for comparison between groups. Total glutathione, GSSG, GSH/GSSG, glutathione reductase, glutathione peroxidase, and ANT redox state were compared between control and diabetic animals with a Student's t test. For all comparisons, the level of significance was established at $P<0.05$.

Results

Animal morphology

Rat body weights, heart weights (corrected for body weight) and fasting levels of glucose and free fatty acids are presented in Table 1. STZ-treated rats had significantly lower body weights as well as lower heart weights when compared to non-STZ-treated rats ($P<0.05$). As expected, STZ-treated rats displayed significantly higher fasting glucose levels when compared to non-STZ-treated rats ($P<0.05$). Circulating non-esterified free fatty acid levels (NEFFA) were not different between control and STZ groups ($P>0.05$). Daily treatment of diabetic animals with MTP-131 had no effect on circulating glucose or NEFFA levels (427±31 mg/dL and 449±73 μM for glucose and NEFFA, respectively; $P>0.05$ compared to pre-treatment levels).

Myocardial glutathione

Myocardial glutathione content is presented in Fig. 2. Although we observed no significant differences in total glutathione content between control and STZ treated rats (Fig. 2A), diabetic animals displayed higher levels of oxidized glutathione (GSSG) and a significant reduction in GSH/GSSG (Figs. 2B and C, respectively; $P<0.05$). We did not observe differences in glutathione reductase or glutathione peroxidase in control versus STZ-treated animals in the study (data presented in Table 1).

Thiol redox state of the ANT

To determine if the oxidized myocardial thiol pool translated to oxidation of the permeability transition pore, we probed the adenine nucleotide translocase (ANT) to determine the redox state of free reactive thiols. There were no differences in the content of ANT protein between control and diabetic animals (Fig. 3A). Fluorescent maleimide labeling revealed that the ANT was more oxidized in diabetic animals compared to controls (Fig. 3B, $P<0.05$ versus control).

Mitochondrial permeability transition pore opening

We found no difference in the quality of our cardiac mitochondria, as reflected by respiratory control ratio. Respiratory control ratios were 8.2±1.5 and 8.7±1.5 in control and diabetic mitochondria, respectively ($P>0.05$). Fluorescence data for Ca-induced PTP opening as well as representative traces is depicted in Fig. 4. Isolated mitochondria from the left ventricle of STZ treated rats required significantly less calcium in order to induce PTP opening when compared to non-STZ treated rats ($P<0.05$), demonstrating that STZ treated rats have an enhanced sensitivity to Ca-induced PTP opening. The enhanced sensitivity to PTP opening in STZ treated rats was significantly decreased ($P<0.05$) when isolated mitochondria were treated with 2 mM dithiothreitol, when animals received daily i.p. injections of 1.5 mg/kg MTP-131 for the 4 days leading up to the experimental day, or when isolated mitochondria were directly treated with 1 nM MTP-131. Treatment with 200 μM diamide significantly increased the sensitivity to Ca-induced PTP opening in non-STZ treated rats ($P<0.05$) but had no effect on Ca-induced PTP sensitivity in STZ treated rats. Treating non-diabetic mitochondria with DTT or MTP-131 (either directly or following daily MTP-131 injections) had no significant effect on calcium retention capacity ($P>0.05$ versus untreated mitochondria; Fig. 4B).

Effects of minocycline and NIM811 on cardiac mitochondria

To validate the pharmacological approaches used in whole-heart studies, we treated isolated mitochondria with minocycline and NIM811. These data are presented in Fig. 5. Incubation of mitochondria with 1 μM minocycline was just as effective at blocking mitochondrial calcium influx as Ru360, a well-characterized mitochondria uniporter antagonist (Fig. 5A). The PTP blocker NIM811 significantly prolonged PTP opening (Fig. 5B), leading to a mean calcium retention capacity of 1067±38 nmol calcium per mg mitochondrial protein. These

data support our use of these compounds in the intact heart to block PTP either directly (with NIM811), or indirectly (with minocycline to reduce mitochondrial calcium influx).

Infarct size

Infarct sizes for hearts in the study are presented in Fig. 6. Hearts from diabetic rats were more susceptible to IR injury, with infarct sizes of 60±4% of the area-at-risk (vs. 46±2% in non-diabetics; $P<0.05$). Administration of 5 μM NIM811, 1 nM MTP-131, or 1 μM minocycline at the onset of reperfusion significantly reduced infarct sizes in both control and diabetic animals. Control hearts treated with NIM811, MTP-131, and minocycline displayed similar reductions that were statistically different from untreated hearts ($P<0.05$, ANOVA). Infarct sizes in diabetic animals were also reduced by NIM811, MTP-131, and minocycline, to levels that were significantly different from the diabetic untreated group ($P<0.05$, ANOVA). Our post-hoc analyses revealed that control animals treated with NIM811, MTP-131, or minocycline were not different from diabetic counterparts treated with the same compound ($P<0.05$ for each post-hoc comparison).

A subset of hearts was perfused with the reducing agent DTT to determine if the beneficial effects of DTT in isolated mitochondria translated to cardioprotection in the intact organ. Interestingly, perfusion with DTT led to potent vasoconstriction that was especially prominent in diabetic animals, with coronary flow decreasing to <3 mL/min g in 50% of diabetic hearts before the onset of ischemia. This robust decline in coronary flow effectively exacerbated the duration of ischemic insult, and led to infarct size expansion to 67±5% of the area-at-risk in diabetic hearts treated with DTT. These findings are consistent with the work of Okazaki et al., where diabetic hearts treated with DTT showed increased ischemia/reperfusion injury and vasoconstriction [93]. Neither the infarct size expansion nor the decline in coronary flow in control animals treated with DTT went to the level of diabetic animals. Infarct size was 58±4%

of the area at risk, and mean coronary flow was 6.1±1.1 mL/min g in non-diabetic hearts treated with DTT prior to ischemia.

Hemodynamics and cardiac arrhythmia

Hemodynamics are presented in Tables 2 and 3. There were no significant differences between groups in left ventricular function or coronary flow before the ischemic period or at the end of the reperfusion protocol ($P>0.05$). Arrhythmia scores are presented in Fig. 7. There were no differences in the incidence of arrhythmias between any of the experimental groups ($P>0.05$).

Discussion

This study was conducted to determine the contributions of mitochondria-derived ROS and mitochondrial calcium to the increased permeability transition and augmented ischemia/reperfusion injury observed in diabetic heart. The major observations from this work are as follows: First, myocardial thiol redox state was more oxidized in the diabetic heart, and this translated to increased oxidation of the adenine nucleotide translocase (ANT), a putative PTP component. Second, the enhanced permeability transition pore (PTP) opening in the diabetic heart was reversed by thiol reductants, and chemical thiol oxidation of diabetic mitochondria had no additional effect. Third, diabetic animals treated with a cell-permeable peptide known to reduce mitochondrial ROS production displayed significant improvements in PTP sensitivity. Finally, the exacerbated cardiac injury in the intact diabetic heart is attenuated to non-diabetic levels with three distinct pharmacological strategies aimed at keeping PTP closed during reperfusion. Taken together, this study provides novel insight into the mechanisms responsible for increased ischemia/reperfusion injury in the diabetic heart.

Enhanced PTP opening in diabetic heart mitochondria

The development of ischemia/reperfusion injury is multi-factorial, and is generally attributed to a combination of tissue necrosis and apoptosis (reviewed in [8]). During the initial phases of ischemia/reperfusion, the majority of tissue death is likely from necrosis [72], and blocking the PTP reduces cardiac necrotic injury in animals (reviewed in [9, 73-75]) and in a recent human clinical trial [94].

Increased susceptibility to cardiac injury in diabetes is evident in both human and animal studies [19, 20, 65-68, 95], and a growing body of literature indicates that augmented PTP opening in the diabetic heart may be (at least partially) responsible for the augmented cardiac injury. Our observation that diabetic rat heart mitochondria display an enhanced propensity for PTP opening corroborates previous work in mice [71], rats [69, 70], and human atrium [66]. While there is a clear role for PTP opening in the diabetic heart, the underlying mechanisms responsible for the augmented PTP opening in the diabetic heart are not fully understood. Among the cellular factors that promote PTP opening, high levels of ROS are known to sensitize the PTP to calcium-induced opening (reviewed in [9, 12, 72, 73, 96]). Myocardial ROS handling is altered in the diabetic state, with diabetic hearts displaying both increased ROS production [20, 97-99] and decreased ROS scavenging [22-24].

Among the anti-oxidant scavenging mechanisms, the reduced/oxidized glutathione couple (GSH/GSSG) is particularly important in mitochondrial physiology (recently reviewed in [100]). The GSH/GSSG couple is the largest capacity thiol buffer in the cell [101], and we previously showed that oxidizing GSH creates instability in mitochondrial membrane potential and evokes cardiac electromechanical dysfunction under normoxic conditions [87]. Our observation that the GSH/GSSG is significantly oxidized in diabetic hearts corroborates studies in human diabetic atrium [102] as well as experimental models of diabetes [68, 78, 79, 103, 104].

Given that decreased GSH/GSSG is known to promote PTP opening [105], we believe that the diabetic heart is characterized by an oxidative shift in the cellular redox environment, which increases PTP open probability due to thiol-dependent modifications of the pore. To determine if PTP thiols are more oxidized in the diabetic heart, we used fluorescently labeled maleimide to determine the thiol redox state of the adenine nucleotide translocase (ANT), a putative PTP component. We found that the ANT is more oxidized in the diabetic heart, supporting the results from our pharmacology experiments in isolated mitochondria. While these are the first findings to demonstrate basal ANT oxidation in the diabetic heart, we must acknowledge that the protein components that combine to form the PTP are still being debated [9, 96], and further studies will be needed to determine if other postulated components of the PTP (such as the phosphate carrier and/or cyclophilin-D) are also oxidized in diabetic myocardium.

A few studies in non-diabetic animals have demonstrated that PTP open probability is augmented with thiol-oxidants and delayed with thiol-reductants [76], ostensibly by redox modulation of pore components [106]. To the best of our knowledge this is the first study to examine the relationship between thiol redox state and PTP opening in the diabetic heart, and provides a crucial link between increased ROS production and PTP opening that are characteristic of the diabetic heart.

We demonstrated that the increased PTP open probability in diabetic mitochondria is reversed with the thiol reducing agent DTT, while DTT had no effect on PTP opening in non-diabetic mitochondria. This corroborates previous findings that treatment with a reducing agent only decreases the sensitivity to Ca-induced PTP opening when conditions of oxidative stress are prevailing [88]. Interestingly, we did not observe a further shift in PTP open probability in diabetic mitochondria treated with diamide, suggesting that PTP thiols are oxidized at rest,

perhaps to a physiological maximum that cannot be surpassed with additional chemical oxidation of the thiol pool.

In addition to the partial rescue of PTP sensitivity that we observed with a thiol reductant, diabetic animals that were treated with a mitochondria-targeted ROS suppressant (either with daily injections or by directly treating isolated mitochondria) also showed significant improvements in mitochondrial calcium retention. MTP-131 belongs to a novel class of cell permeable small peptides that selectively target mitochondria and localize at the inner mitochondrial membrane. MTP-131 is known to reduce mitochondrial ROS levels in several different models and protect against cell death induced by a variety of chemical and biological stressors (reviewed in [3, 80]).

Our demonstration that MTP-131 improved PTP sensitivity in the diabetic heart is novel, and provides strong evidence that mitochondria-derived ROS in the diabetic heart causes shifts in the thiol redox state, ultimately leading to increased PTP opening. Our data suggest that MTP-131 is not a direct PTP blocker, as calcium retention capacity in control animals was not affected by this compound (contrast with NIM811, see Figs. 4 and 5). Rather, it appears that MTP-131 is acting to lower ROS levels in vivo, ostensibly keeping PTP thiols in a more reduced state by maintaining GSH/GSSG. While speculative, these data are in agreement with previous studies showing better maintenance of the thiol redox pool in animals treated with the MTP-131 analog SS-31 [107]. This idea is further supported by observations that ROS production is minimal when mitochondria are respiring on glutamate/malate [108], implying that MTP-131 should have no effect on PTP opening if an oxidant burden is not present.

Interestingly, neither the thiol-reductant nor MTP-131 restored PTP sensitivity to non-diabetic levels. Several possibilities could explain these findings. First, the expression of putative

pore components (specifically, cyclophilin-D) is elevated in diabetic hearts [71, 109], which may confound our results since more cyclophilin-D protein is available to associate with the pore. Second, in addition to the pore regulation by calcium and ROS, ADP and pH also regulate PTP opening. Several studies have found differences in mitochondrial energetics in the diabetic heart [68], which may explain why we did not fully reverse the PTP sensitivity. We noted no difference in the quality of our mitochondrial preparations (as reflected by similar respiratory control ratios) or the state IV respiratory rates, indicating that differences in uncoupling were likely not present in our model.

Decreasing reperfusion injury by targeting cardiac mitochondria

Our data in isolated mitochondria indicated a clear difference in the susceptibility to PTP opening between non-diabetic and diabetic animals. These findings are in agreement with our observations in intact hearts, where we saw a significantly greater extent of ischemia/reperfusion injury in diabetic myocardium. Infarct sizes in diabetic hearts were approximately 30% larger than non-diabetic counterparts, which is in general agreement with a number of studies in the literature indicating that diabetic hearts are more susceptible to ischemia/reperfusion injury [110-115]. We conducted studies in the intact heart to determine if approaches that diminish the open probability of the PTP salvaged cardiac tissue. We used 3 compounds targeted at heart mitochondria to decrease reperfusion injury: direct inhibition of the PTP with NIM811, and indirect inhibition by keeping matrix calcium and ROS low with minocycline and MTP-131, respectively. To increase the clinical relevance of our findings we only administered the compounds at the onset of reperfusion (as opposed to pre-treatment, which has limited clinical application [116]). Administration of NIM811, MTP-131, or minocycline reduced infarct size in non-diabetic animals, with no differences in the extent of cardioprotection between the compounds. The infarct-salvaging that we observed in our non-diabetic animals corroborates

previous studies in the literature where these treatments reduced ischemia/reperfusion injury in otherwise healthy animals [3, 117, 118].

Directly blocking the PTP in diabetic hearts

In our whole heart experiments, we directly blocked the PTP with NIM811, an analog to cyclosporin-A that shows greater specificity for cyclophilin D. A number of studies have shown that blocking the PTP with cyclosporin-A decreases infarct size [75, 77, 81, 119, 120], including recent studies in human clinical trials [94]. Although promising, the use of CsA is confounded by several problems, including a narrow therapeutic window [121], potentially harmful effects to the microvasculature [122-124], deleterious effects on long-term myocardial function [125], suppression of mitochondrial respiration [81], nephrotoxicity [126], and immunosuppression [127]. Given that diabetics are known to have high rates of microvascular dysfunction [128], both cardiac and mitochondrial dysfunction [68], renal failure [129] and depressed immune function [130], cyclosporin-A may be contraindicated in this patient population. Accordingly, we employed NIM811 to block PTP opening in isolated hearts. NIM811 is a non-immunosuppressive derivative of cyclosporin-A and is very effective at blocking PTP across a wider range of doses [81, 131]. NIM811 blocks PTP formation by selectively binding matrix cyclophilin-D, but unlike cyclosporin-A it does not bind cyclophilin A [131]. Our finding that NIM811 protected diabetic hearts from injury is in agreement with several previous studies showing infarct size reduction with NIM811 [81, 117], although this is the first study to demonstrate its efficacy in a diseased population.

Indirectly blocking PTP by targeting mitochondrial calcium and ROS

Given that PTP open probability is promoted by calcium and ROS [9, 12, 72, 96], we used two novel agents targeted at mitochondrial calcium and ROS to decrease reperfusion injury in diabetic hearts by indirectly reducing the open probability of PTP. Calcium entry into the

matrix occurs through the mitochondrial calcium uniporter [52]. Both ruthenium red and Ru360 block mitochondrial calcium influx through the uniporter [132, 133], but these compounds have nonspecific effects on intracellular calcium transients [132, 134]. Furthermore, while effective in isolated mitochondrial studies, Ru360 appears to be impermeable across cell membranes, precluding therapeutic use [135, 136]. We used the tetracycline anti-biotic minocycline to block matrix calcium influx. The ability of minocycline to block mitochondrial calcium fluxes was recently demonstrated in liver mitochondria [82], and our data herein demonstrate that minocycline is just as effective as Ru360 at blocking mitochondrial calcium influx in cardiac mitochondria. Minocycline is highly permeable to heart cells [118], and has no discernable effect on myocyte calcium handling or baseline cardiac hemodynamics [137], making it an attractive candidate to modulate mitochondrial calcium fluxes in the intact heart/animal. In our study, blocking mitochondrial calcium influx at the onset of reperfusion was just as effective at salvaging tissue as directly blocking the PTP. Some studies have suggested that minocycline depolarizes cardiac mitochondria, but only at concentrations at least two orders of magnitude higher than used herein [138, 139]. Indeed, we found no effect of minocycline on mitochondrial membrane potential (RC Sloan and DA Brown, unpublished observations using triphenylphoshonium uptake). This suggests that the protective effects observed in our study are due to inhibiting calcium influx, and not by an uncoupling mechanism. To determine if indirectly inhibiting PTP opening by lowering mitochondrial ROS levels decreased reperfusion injury in diabetic hearts, we used the novel cell permeable compound MTP-131. Our observation in diabetic animals that lowering mitochondrial ROS levels at reperfusion reduces infarct size is novel. Although this is in general agreement with other studies where over-expression of anti-oxidants protected diabetic hearts against post-ischemic injury [140, 141], we did not observe differences in functional recovery in any of our drug-treated groups herein. Furthermore, we saw

no beneficial effects on the incidence of cardiac arrhythmia with any of the strategies targeting PTP, providing further evidence that PTP blockers do not markedly influence the incidence/severity of reperfusion arrhythmia (recently reviewed in [142]). Given that the thiol-reductant DTT partially reversed the PTP sensitivity in isolated diabetic mitochondria, we also administered DTT to intact hearts to determine if this translated to infarct salvage. Interestingly, we observed significant vasoconstriction in diabetic animals that were treated with DTT prior to ischemia, which led to expansion of the infarct size. These effects of DTT on diabetic hearts are in agreement with another recent study where DTT reduced basal coronary flow by ~40% and increased infarct size in diabetic hearts [93], and suggest that the reduction in coronary flow in diabetic animals exacerbated the extent of injury in diabetic animals. Although the mechanisms underlying DTT-dependent vasoconstriction in diabetics are beyond the scope of our study, it is tempting to speculate that DTT may be disrupting the bioavailability of nitric oxide by interfering with other thiol-dependent signaling (such as S-nitrosylthiol modifications) in the coronary endothelium. Clearly further study is warranted to fully understand the effects of DTT in the diabetic vasculature.

Study limitations

One limitation to our study is that streptozotocin was used to induce diabetes. Although this model more closely resembles Type I diabetes, there are a number of similarities to Type II diabetes models. Similar to both humans and animal models of type 2 diabetes, hearts from STZ-induced diabetic rats display alterations in myocardial calcium handling, substrate utilization, mitochondrial energetics, and enhanced oxidative stress (recently reviewed in [19]).

Conclusions

In summary, these experiments provide evidence that augmented reperfusion injury observed in the diabetic heart is a consequence of enhanced susceptibility to mitochondrial

permeability transition. Diabetic hearts displayed oxidative shifts in the myocardial glutathione pool, and this translated to increased oxidation of PTP components, namely the ANT. Increased PTP opening in diabetic heart mitochondria was partially reversed either with a thiol-reductant or daily treatment with a mitochondria-targeting peptide, suggesting that greater ROS burden in the diabetic heart shifts the intracellular redox environment and promotes PTP opening. In the whole heart, three novel pharmacological approaches at the onset of reperfusion significantly decreased injury, suggesting that either direct or indirect inhibition of PTP has significant clinical potential to reduce the burden of ischemic heart disease among the rapidly growing diabetic population.

Table 1

Animal morphology and baseline biochemical parameters. Data are presented as mean ± SEM.

	Control	STZ
Body weight (g)	348 ± 6.8	307 ± 12.4*****
Heart weight/body weight (mg/g)	5.29 ± 0.12	4.44 ± 0.17*****
Fasting glucose (mg/dL)	114 ± 1.4	495 ± 21.9*****
Circulating non-esterfied free fatty acids (μM)	448 ± 63	390 ± 62
Glutathione reductase activity (U/g protein)	70 ± 13	55 ± 7
Glutathione peroxidase activity (U/g protein)	452 ± 43	345 ± 80

*****$P<0.05$ versus control.

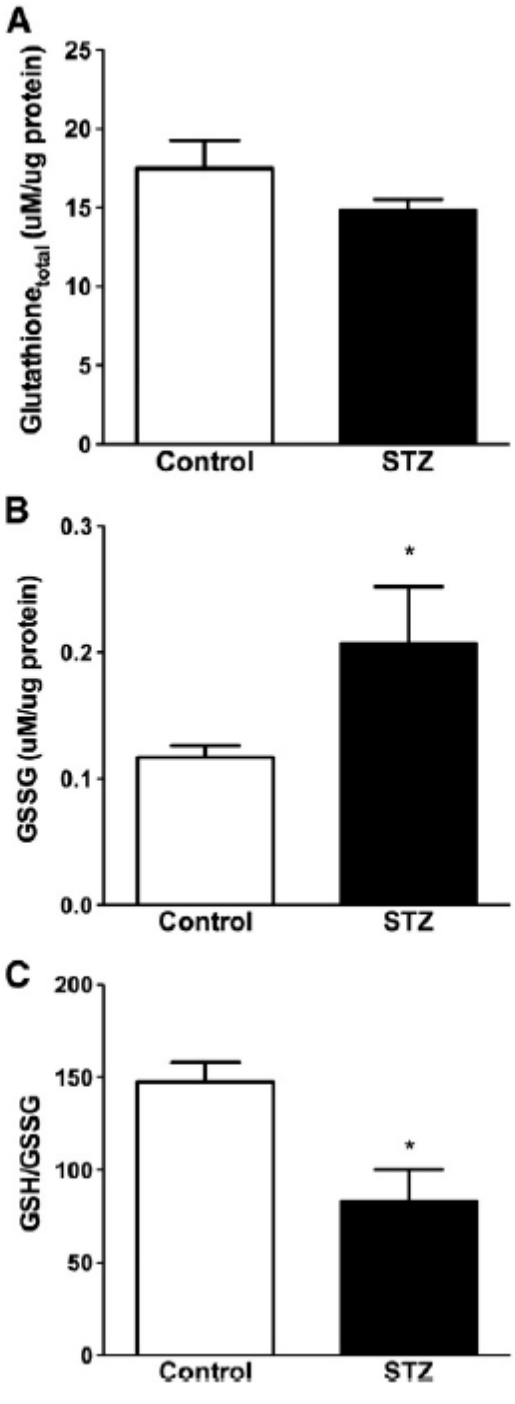


Figure 2. Glutathione content in the diabetic heart. **A**. Total glutathione in control and diabetic (STZ) left ventricles. **B**. Oxidized glutathione (GSSG) content from control and diabetic (STZ) hearts. **C**. Ratio of reduced to oxidized glutathione (GSH/GSSG) in diabetic hearts in the study. **P<0.05* versus control.

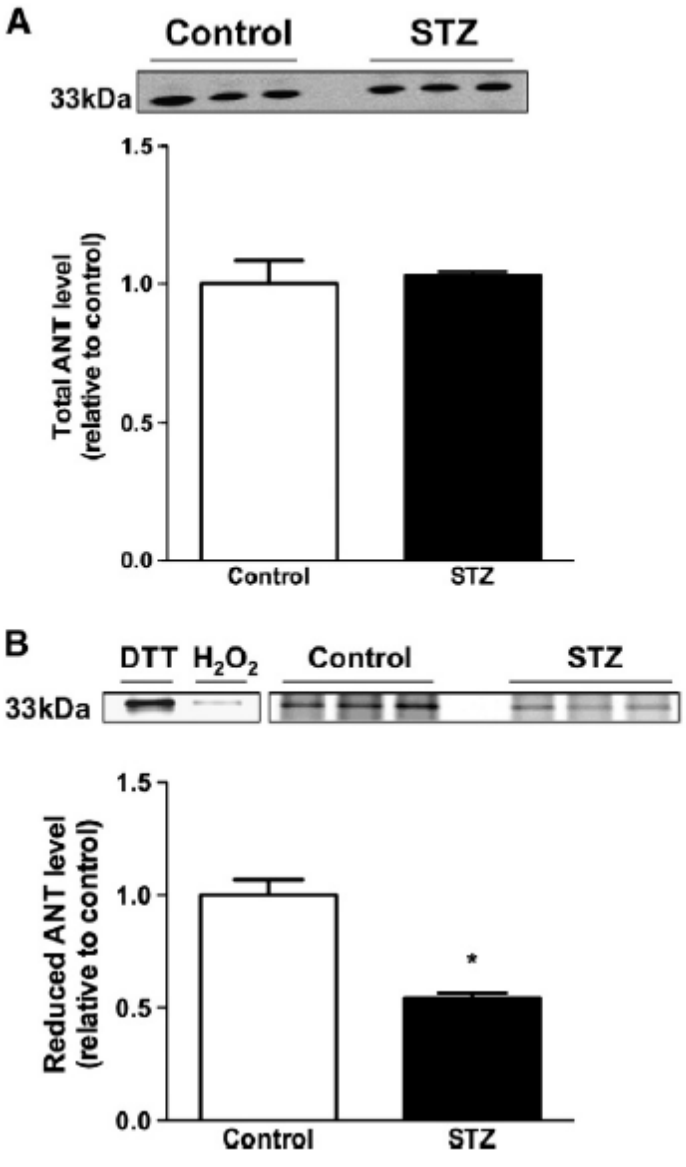


Figure 3. Adenine nucleotide translocase (ANT) content and redox state in hearts from the study. **A**. Total content of ANT in hearts from the study using Western blot. **B**. Levels of reduced thiols on the ANT assessed using immunoprecipitation and serial maleimide labeling to fluorescently label reduced \SH groups. Confirmation of the assay sensitivity is provided where the thiol reductant dithiothreitol (DTT) and hydrogen peroxide (H_2O_2) led to ANT reduction and oxidation, respectively. *$P<0.05$ versus non-diabetic.

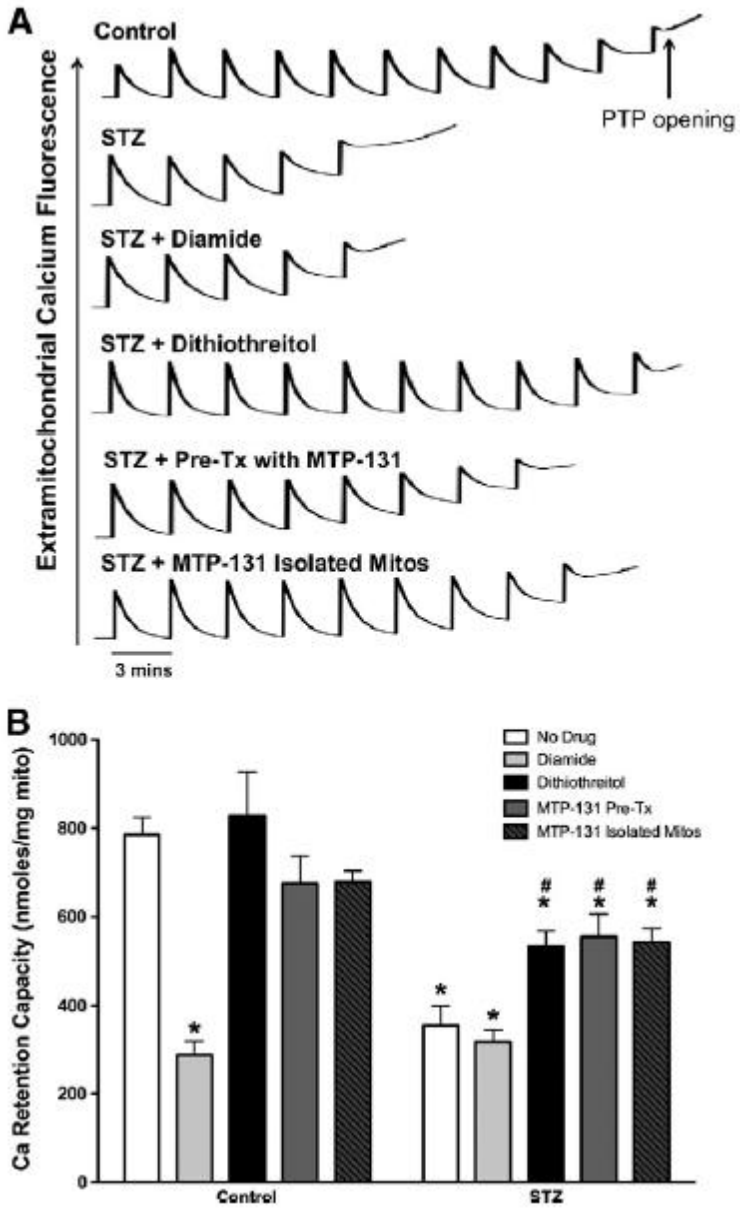


Figure 4. Influence of thiol redox modifications on Ca-induced opening of the permeability transition pore in isolated cardiac mitochondria. **A**. Representative extramitochondrial calcium fluorescence traces from diabetic mitochondria following 50 nmol pulses of $CaCl_2$ to induce permeability transition. **B**. Quantification of calcium retention capacity in isolated mitochondria from non-diabetic and diabetic animals. Mitochondria were either treated with diamide (thiol oxidizing agent) or dithiothreitol (DTT; thiol reducing agent). In other studies we examined the effect of the mitochondria-targeting ROS suppressant MTP-131. Animals were either pre-treated with MTP-131 for 4 days prior to isolated mitochondria experiments (MTP-131 Pre-Tx), or the effect of MTP-131 on calcium retention capacity was directly examined by treating mitochondria with 1 nM MTP-131 for 10min prior to beginning calciumpulses. *$P<0.05$ versus non-drug treated control; #$P<0.05$ versus non-drug treated STZ; n=6–14 calcium retention experiments in each group. Abbreviations: STZ, streptozotocin-induced diabetes; PTP, mitochondrial permeability transition pore.

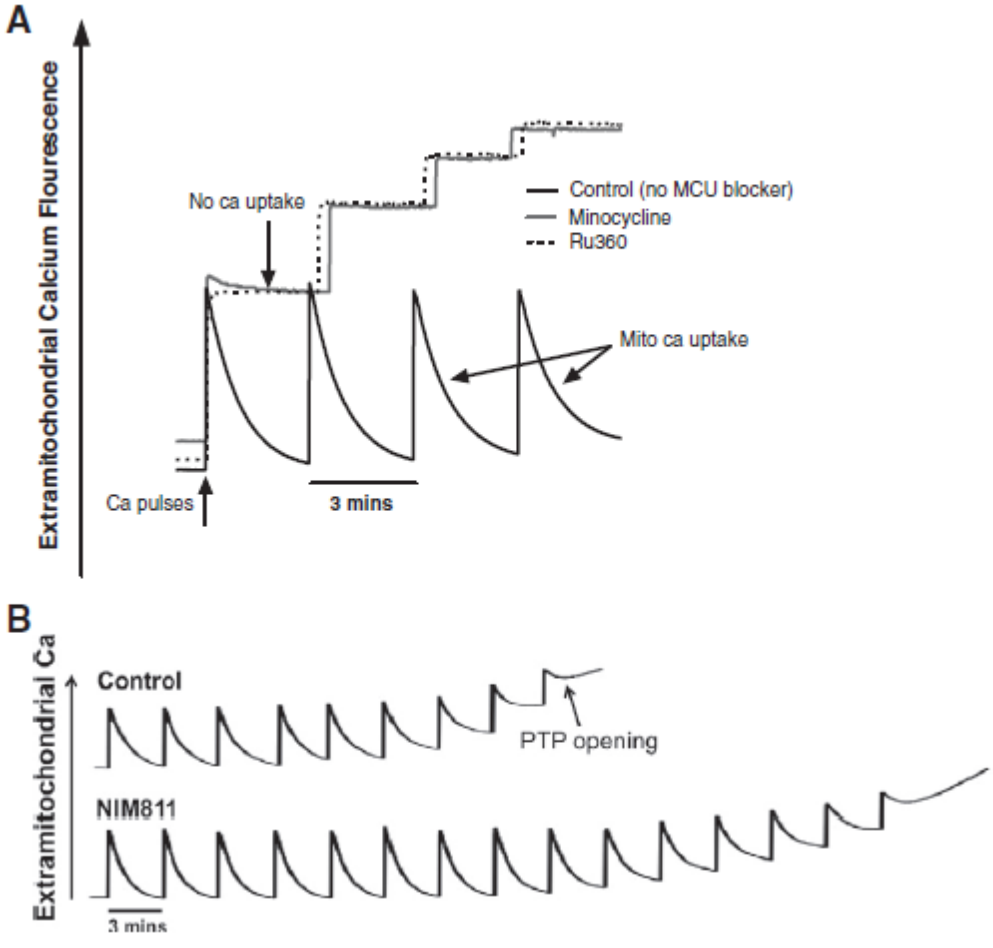


Figure 5. Influence of compounds used in whole-heart studies on calcium uptake and PTP opening in isolated cardiac mitochondria. **A**. Extramitochondrial traces showing that the tetracycline anti-biotic minocycline (1 μM) is just as effective as Ru360 in inhibiting mitochondrial calcium uptake in isolated cardiac mitochondria. **B**. Representative traces indicating delayed PTP opening in mitochondria treated with 5 μM NIM811 (mean calcium retention was 1067±38 nmol calcium per mg mitochondrial protein). Abbreviations: STZ, streptozotocin-induced diabetes.

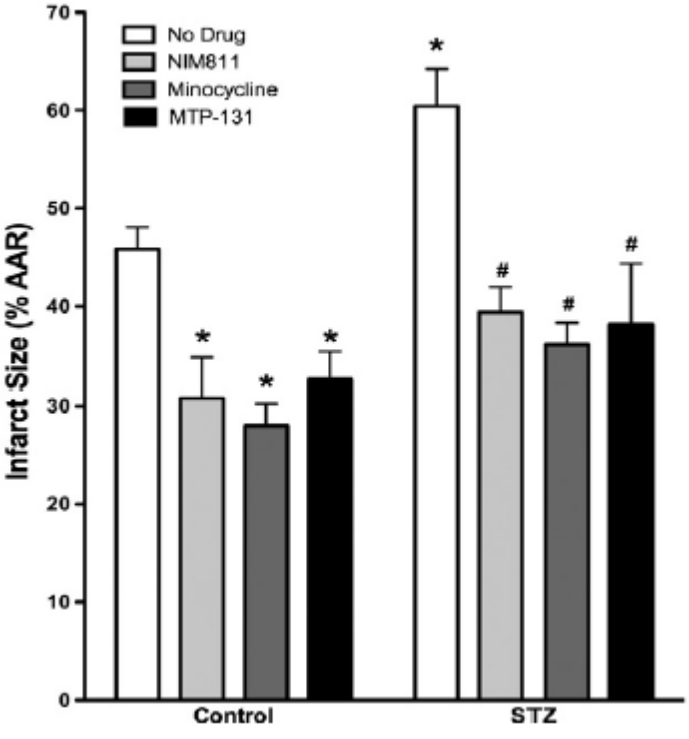


Figure 6. Infarct sizes in hearts in the study. Data are presented as mean±SEM; **P<0.05* versus control; *#P<0.05* versus non-drug treated STZ; n=5–10 in each group. All compounds were administered at the onset of reperfusion. Abbreviations: STZ, streptozotocin-induced diabetes.

Table 2

Baseline hemodynamics for hearts in the study.

	Control	STZ
LVDP (mmHg)	123 ± 12	119 ± 9
+dP/dt (mmHg/s)	4312 ± 444	3580 ± 367
-dP/dt (mmHg/s)	-2791 ± 223	-2266 ± 254
Coronary flow (mL/min/g heart wet wt)	8.3 ± 0.7	8.8 ± 1.5

Abbreviations; LVDP, left ventricular developed pressure; +dP/dt, maximal rate of contraction; -dP/dt, maximal rate of relaxation; n = 5-10 in each group.

Table 3

Cardiac hemodynamics at the end of the 2 hour reperfusion in control and diabetic (STZ) rat hearts.

	LVDP (mmHg)	+dP/dt (mmHg/s)	-dP/dt (mmHg/s)	Coronary flow (mL/min g wet wt)
Control	30 ± 7	768 ± 96	-535 ± 63	3.8 ± 0.4
Control + NIM811	22 ± 3	734 ± 101	-518 ± 73	2.5 ± 0.3
Control + Minocycline	36 ± 6	1239 ± 195	-786 ± 101	4.1 ± 0.2
Control + MTP-131	34 ± 4	1069 ± 110	-704 ± 63	3.7 ± 0.2
STZ	26 ± 3	816 ± 126	-489 ± 63	4.3 ± 0.7
STZ + NIM811	25 ± 3	703 ± 117	-456 ± 57	3.7 ± 0.2
STZ+ Minocycline	32 ± 9	758 ± 173	-456 ± 57	4.3 ± 0.8
STZ + MTP-131	24 ± 4	789 ± 174	-475 ± 75	4.0 ± 0.6

Abbreviations; LVDP, left ventricular developed pressure; +dP/dt, maximal rate of contraction; -dP/dt, maximal rate of relaxation; all pharmacological compounds were administered beginning at the onset of reperfusion. n = 5-10 in each group.

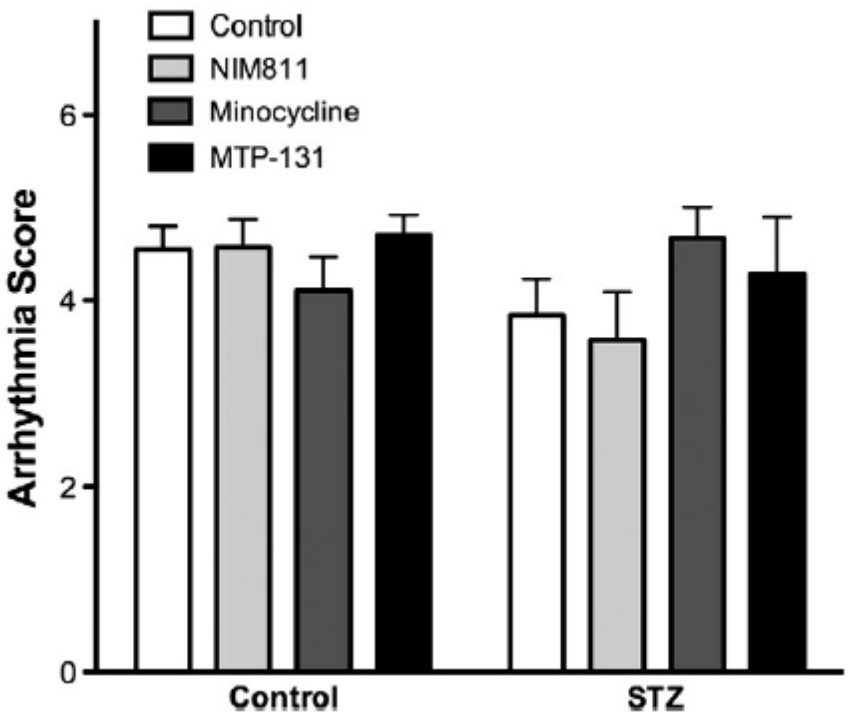


Figure 7. Quantification of arrhythmia scores from isolated rat hearts following 20 min of ischemia and 120 min of reperfusion. Data are presented as mean±SEM. There were no significant differences between groups, N=5–10 in each group. Abbreviations: STZ, streptozotocin-induced diabetes.

Chapter 3: The mitochondria-targeting peptide Bendavia restores mitochondrial function in diabetic hearts by improving mitochondrial calcium handling and oxidative phosphorylation

Introduction

Diabetic cardiomyopathy is one of the leading causes of heart failure in diabetic patients; however, effective treatment for this disease is still lacking. Most medical care for diabetic patients focuses on glycemic control despite the fact that 60% of these patients with acceptable glycemic control and with no clinically detectable heart disease still show signs of a dysfunctional myocardium when screened with Valsalva maneuver and pulmonary venous recording [143, 144]. Therapies targeting the dysfunctional diabetic myocardium, especially in the early asymptomatic stages, that can reduce the progression to heart failure are currently lacking.

Recent evidence suggests that decrements in bioenergetics coordination are centrally involved in diabetic cardiomyopathy. Indeed, while the *healthy* heart is proficient at adjusting its energetic supply to demand, the diabetic heart has a very limited capacity of energy matching [17, 18]. A growing body of literature indicates that alterations in mitochondrial function may be responsible for the increased cardiac dysfunction observed in diabetes (reviewed in [19]).

A key signaling molecule in sustaining adequate cardiac function with changing metabolic demand is Ca. The mitochondrial uptake of Ca is essential for adjusting cardiac function to changing metabolic needs, since Ca stimulates oxidative phosphorylation by activating several enzymes of the Krebs Cycle as well as the ATP-synthase of the respiratory chain [46-51].

Ca entry into mitochondria occurs through the mitochondrial Ca uniporter (MCU), an ion channel within the inner mitochondrial membrane whose molecular identity has recently been elucidated [52, 53], and this process is regulated by at least 2 newly characterized mitochondrial proteins: Mitochondrial Ca uptake 1 (MICU1) [54] and mitochondrial Ca uptake regulator 1 (MCUR1) [55]. While it is well known that mitochondrial calcium influx is decreased in the diabetic heart [57-59], there are no data relating this decrease to changes in the mitochondrial proteins handling Ca uptake. Accordingly, whether these proteins can be regulated by a therapeutic intervention is not known.

Therapies that can improve overall mitochondrial function in the diabetic heart have clear potential to mitigate the burdens associated with diabetic cardiomyopathy. Bendavia is a cell-permeable mitochondria-targeting peptide being currently tested in clinical trials for cardiovascular and renal disease. We recently showed that Bendavia is able to delay the opening of mitochondrial permeability transition pore, and reduce ischemia reperfusion injury in diabetic hearts [62].

The aim of this study was to determine if daily Bendavia treatment could result in improved mitochondrial function by: 1) reversing the decrement in mitochondrial calcium uptake by regulating the levels of mitochondrial Ca uptake proteins, 2) ameliorating the oxidative phosphorylation machinery, and 3) improving the mitochondrial respiratory capacity.

Methods

Materials

Unless otherwise noted, all reagents used were obtained from Sigma-Aldrich. Ca green 5 N salt probe and Amplex Ultra Red Reagent (AUR) were purchased from Invitrogen (Carlsbad, CA, USA), Bendavia was obtained from Stealth Peptides Inc.

Experimental animals

All animal procedures received prior approval by the East Carolina University Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (7–9 weeks old) were housed in a temperature (22°C) and light-controlled (12 hour light/12 hour dark) environment and fed standard rat chow (Research Diets, New Brunswick, NJ, USA) and water ad libitum. After 5 days of acclimation to the facility, diabetes was induced with a single intraperitoneal (i.p.) injection of streptozotocin (STZ, 65 mg/kg) dissolved in 100 mM sodium citrate (pH=4.5) following a 12-hour overnight fast. Control animals received an i.p. injection of sodium citrate. All experiments were performed 4 weeks following STZ injection. Blood glucose was determined using blood from tail vein and a commercially available glucometer (One Touch Ultra 2, LifeScan, Milpitas, CA, USA). On the day of experimentation, rats were anesthetized with a ketamine/xylazine cocktail (90 mg/kg and 10 mg/kg, respectively; i.p. injection) and hearts were removed via bilateral thoracotomy, and studied on Langendorff, used to prepare skinned cardiac fibers, isolated mitochondria, or flash frozen and later used for western blots. The hearts used for Langendorff studies were collected at the end, dried out and the dry weight used to normalize the myocardial oxygen consumption.

In vivo treatment with Bendavia

Animals received an intra-peritoneal injection of either Bendavia (1.5 mg/kg in 0.9% saline) if they were in the control-Bendavia and STZ-Bendavia groups, or an equivalent volume of saline if they were in the groups of control-saline and STZ-saline. These injections were given daily during the last 4 days post-STZ/citrate injection and prior to studying the animals at 4 weeks post STZ-injection.

Isolated Heart Studies

For whole-heart studies, rat hearts were perfused (perfusion pressure of 75 mmHg) in a retrograde fashion on a modified Langendorff apparatus using an established protocol by our group [62, 87]. Hearts were perfused with a modified Krebs-Henseleit buffer containing (in mM): 118 NaCl, 24 $NaHCO_3$, 4.75 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.0 $CaCl_2$, and 10 glucose (gassed with 95/5% O_2/CO_2). Hearts were bathed in a buffer-filled perfusion chamber maintained at 37°C for the duration of the experiments. Following the initiation of perfusion, hearts were instrumented for the simultaneous evaluation of mechanical and electrical function. A buffer-filled latex balloon (size 5, Harvard Apparatus, Holliston, MA, USA), calibrated at the beginning of each day using a digital manometer, was inserted into the left ventricle (via the mitral valve) for the measurement of left ventricular developed pressure (LVDP), with balloon volume adjusted to establish a diastolic pressure of 5-8 mmHg. Three electrodes were placed into the buffer-filled perfusion chamber for the measurement of volume-conducted ECG. Coronary flow rates were monitored throughout the experiment with a flow probe (Transconic Systems, Ithaca, NY, USA) connected in series with the perfusion line. All hemodynamic parameters were continuously monitored and stored on a personal computer using commercially available software (Chart, AD Instruments, Colorado Springs, CO, USA). Heart rate was calculated using the LVDP trace.

Energy supply-demand matching protocol

Following a 20 min equilibration and a 10 minute baseline period, the hearts were paced for 3 minutes at 5 Hz then for 3 minutes at 7 Hz. The pacing was stopped and the hearts received a bolus of 5μM carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) via an injection port to evaluate the maximal mitochondrial respiratory capacity. The wet weight of the whole heart, and the left ventricle were recorded and the whole cardiac tissue was dried for 24h to get the heart's dry weight which was used to normalize the myocardial oxygen consumption.

Preparation of skinned cardiac fibers

Skinned cardiac fibers were prepared according to a method described by Anderson and Neufer (2006) [145]. Following the heart excision from anesthetized animals, the left ventricle was dissected and placed on a petri dish containing ice-cold buffer X containing in (mM): 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 20 Taurine, 5.7 ATP, 14.3 Phosphocreatine, 6.56 $MgCl_2$-$6H_2O$ and 50 MES; pH 7.1. All fat and connective tissue was removed under a dissecting microscope, and small cardiac fiber bundles (5-7 mg of wet weight) were prepared. Fiber bundles were permeabilized using 50µg/ml saponin at 4°C for 20 min. Permeabilized fiber bundles were then washed, 3 times for 5 min each, in ice-cold buffer Z containing in (mM): 110 K-MES, 35 KCL, 1 EGTA, 5 K_2HPO_4, 3 $MgCl_2$-$6H_2O$, and 5 mg/ml BSA; pH 7.4. The fiber bundles were kept at 4°C in buffer Z until analysis.

Analysis of mitochondrial function in skinned cardiac fibers

All experiments were conducted at 37°C in presence of 20 µM blebbistatin to prevent contraction as previously described [146]. We used the Oroboros system (Oroboros Instruments, Innsbruck, Austria) to measure O_2 consumption. Buffer Z was used as respiration medium and O_2 consumption was monitored during a substrate-inhibitor titration protocol in order to obtain a step-by-step analysis of various components of the mitochondrial respiratory chain as described by Kuznestov et al. (2008) [147]. The addition of substrates and inhibitors was done in the following sequence: Glutamate/malate (10mM/5mM), ADP (2 mM), Rotenone (0.5 µM), succinate (10 mM), antimycin A (5 µM), N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)/ascorbate (0.5mM/2mM), and finally cytochrome C (10 µM). At the end of each experiment the fiber bundles used were washed with distilled water and lyophilized in a freeze-dryer (Labconco, Kansas city, MO) for 3 hours and then weighed on a microsclae (Mettler-Toledo XS3DU). O_2 consumption is expressed in pmol/min*mg dry wt.

Cardiac mitochondria isolation

Cardiac mitochondria were isolated from the LV following our established protocol [62], and all steps were performed at 4°C. Briefly, after rinsing the excised heart in ice-cold isolation solution (IS) containing 300mM sucrose, 10 mM sodium-hepes and 0.2 mM EDTA, pH 7.2, the LV was minced into small pieces, digested with trypsin (0.125mg/ml IS) for 2 minutes. The digestion was then stopped with soybean trypsin inhibitor (0.65 mg/ml IS) containing 1 mg/ml BSA. The buffer was removed, and the tissue resuspended in IS/BSA, and homogenized with a teflon Potter homogenizer. The homogenate was centrifuged at 600 x g for 10 min, and the resultant supernatant was centrifuged at 8,000 x g for 15 min. The supernatant was discarded, and the pellet re-suspended in IS/BSA. This step was repeated one more time, and the final pellet was resuspended in a small volume of IS, and kept on ice until use.

Calcium uptake studies

Quality of mitochondria was assessed by measuring their coupling state (measure of respiratory control ratio; RCR). State 2 (non-phosphorylating state) and state 3 (phosphorylating state) respiration were measured in buffer Z using the high resolution OROBOROS O_2K oxygraph. The assays were conducted at 25°C under continuous stirring, and state 2 respiration was initiated by the addition of glutamate (10 mM) and malate (5mM). Once oxygen consumption reached a steady state, saturating level of ADP (2.5 mM) was added to initiate state 3 respiration. The RCRs were calculated by dividing state 3 respiration by state 2 respiration. Mitochondrial Ca uptake studies were performed as described in Sloan et al. 2012 [62]. Briefly, 0.75 mg mitochondrial protein were suspended in 2ml assay buffer (125mM KCl, 5mM HEPES, 2mM KH_2PO_4, 1mM $MgCl_2$, pH = 7.3) containing 1 μM of the fluorescent probe Ca green 5 N salt (506 and 532 nm excitation and emission wavelengths respectively), and extra-mitochondrial Ca fluorescence was monitored with a fluorescence spectrophotometer (Photon Technology

International, Birmingham, NJ, USA), following pulses of 50 nmoles $CaCl_2$. All uptake studies were performed under state 2 conditions (5 mM glutamate/5 mM malate), and time to 63% corresponded to the time it took the mitochondria to accumulate 63% of total Ca accumulated following 1 pulse of $CaCl_2$. For experiments where redox state was manipulated, energized mitochondria were treated with 2 mM of the thiol reductant dithiothreitol (DTT) 10 min before initiating Ca pulses.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential ($\Delta\Psi m$) was determined using a method similar to that described by Kamo et al. [148]. Mitochondrial accumulation of the lipophilic cation, tetraphenylphosphonium (TPP^+) was monitored with a TPP^+ selective electrode and an Ag/AgCl reference electrode (World Precision Instruments, Sarasota, FL, USA) as changes in voltage. The latter was monitored on a Dell computer using commercially available software (Chart, AD Instruments, Colorado Springs, CO, USA). The $\Delta\Psi m$ was estimated from the equation: $\Delta\Psi m = 58*\log(v/V) - 58*\log(10^{F\Delta E}/^{2.3RT}-1)$ [148], where ΔE is deflection in TPP^+ voltage from baseline; R is gas constant; T is temperature; v is mitochondrial matrix volume, and V is buffer volume. The baseline voltage was taken before addition of substrates, and mitochondrial matrix volume was assumed to be of 1µl/mg protein [149]. Based on a previous study [150], we assumed that matrix volumes were similar between diabetic and non-diabetic rats. For $\Delta\Psi m$ experiments, both the TPP^+ selective electrode and the reference electrode were placed in a magnetically stirred chamber containing 2.5 ml mitochondria assay buffer (described above), 0.5 mg mitochondria, supplemented with 1.2 µM TPP^+, and glutamate/malate (5 mM each). State 3 respiration was initiated with the addition of 2.5 mM ADP.

Western Blot analysis

Cardiac LV mitochondria were isolated as described above, and protein content was determined using a BCA protein assay. Aliquots of LV mitochondria proteins were solubilized, subjected to SDS-PAGE and transferred to an Immobilon-P transfer membrane. After blocking in Tris-buffered saline containing 0.1% Tween (TBS-T) and 4% bovine serum albumin for 1 h at room temperature, the membrane was incubated with the mitochondrial Ca uniporter (MCU) antibody (1:1000; Santa Cruz), mitochondrial Ca uptake 1 (MICU1) antibody (1:1000, abcam), mitochondrial Ca uniporter regulator 1 (MCUR1) antibody (1:1000, Aviva Systems Biology), total OXPHOS cocktail antibody (1:500; MitoSciences), or VDAC (1:1000, abcam) overnight at 4 °C. The membrane was washed with TBS-T and incubated with an IR-Dye-conjugated secondary antibody for 1 h at room temperature then washed again with TBS-T and finally with TBS. The immunoreactive bands were scanned and quantified using the Odyssey Infrared Imaging system and software, respectively (LI-COR Biosciences, Lincoln, NB).

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using a two way or a one way ANOVA with Tukey's multiple comparison post-hoc analysis. For all comparisons, the level of significance was established at $P<0.05$.

Results

Effect of Bendavia on morphological, metabolic and hemodynamic parameters in diabetic rats

Morphological, metabolic and hemodynamic data are presented in Table 1. Animals in the STZ-saline group were hyperglycemic as expected, and showed a significant decrease in body and heart weights when compared to non-diabetic animals in the control-saline and control-Bendavia groups ($P<0.05$). The decrease in heart weight was associated with a significant decrease in both LV and RV mass, and although the decrease was statistically significant when

normalized to body weight and in comparison to non-diabetic animals (*P<0.05*), this difference was no longer significant once normalized to tibia length (*P>0.05*). In the isolated perfused heart studies, the left ventricular developed pressure at baseline was not significantly different among groups. Acute treatment with Bendavia didn't have any significant effect on the morphological parameters (*P>0.05*).

Effect of Bendavia on cardiac bioenergetics

In isolated perfused hearts

Assessment of myocardial oxygen consumption in isolated hearts shows that diabetic hearts have a significantly decreased MVO_2 at baseline, as well as in response to increased cardiac workloads (pacing at 5 Hz and 7 Hz), and that treatment with Bendavia significantly improves their MVO_2 under all conditions as it is shown in STZ-Bendavia group ($P<0.05$; Fig. 8A). Furthermore, the rate of increasing MVO_2 in response to increased cardiac workloads is significantly slower in the diabetic hearts and is increased to control levels following treatment with Bendavia at 5Hz but not at 7 Hz (Fig. 8B and C).

In skinned cardiac fibers

Mitochondrial respiratory capacity was overall decreased in cardiac fibers from diabetic hearts, and improved following treatment with Bendavia. Under state 2 condition (no ADP), complex I-supported respiration was significantly decreased in diabetic cardiac fibers, and treatment with Bendavia restored it to control levels, as it is shown in STZ-Bendavia group (*P<0.05*; Fig. 8D). Under state 3 conditions (2.5 mM ADP), complex I-supported respiration was decreased in diabetic cardiac fibers even though it didn't reach statistical significance, and was improved with Bendavia treatment (Fig. 8E). Under state 3 conditions, complex II-supported respiration was significantly decreased in diabetic cardiac fibers, and significantly improved by

the treatment with Bendavia ($P<0.05$; Fig. 8F). Complex IV-supported respiration in the presence of ADP was not significantly different among groups (data not shown).

Effect of Bendavia on mitochondrial function

Mitochondrial maximal respiratory capacity

Maximal mitochondrial respiratory capacity, as assessed with the uncoupler FCCP in isolated perfused hearts, did not show any significant difference among groups (*P>0.05*; Table 1).

Mitochondrial Ca uptake

Ca uptake was significantly slower in cardiac mitochondria from diabetic animals compared to mitochondria from control animals (*P<0.05*), and the treatment with Bendavia led to a significant increase in the ability of diabetic mitochondria to uptake Ca as illustrated in Fig. 9A (*P>0.05*; STZ-Bendavia group). Pre-treating cardiac mitochondria from diabetic hearts with the thiol-reducing agent DTT had a similar effect (*P<0.05*; Fig. 9B and C).

Mitochondrial membrane potential

Mitochondrial membrane potential, as assessed with TPP in isolated mitochondria, did not shown any significant difference among groups (*P>0.05*; Fig. 9D).

Effect of Bendavia on the expression level of mitochondrial Ca handling proteins

Mitochondria from diabetic hearts showed a significant decrease in the expression level of both MCU and its regulatory protein MCUR1, and treatment with Bendavia re-established the expression level of both proteins to control levels (Fig. 10A and C). The other regulatory protein of MCU (MICU1) was decreased following treatment of diabetic rats with Bendavia but this change did not reach a statistical significance (Fig. 10B).

Effect of Bendavia on the expression level of oxidative phosphorylation proteins

Mitochondria from diabetic hearts showed a significant decrease in the expression level of both complex I and complex V, and the treatment with Bendavia re-established the expression level of both proteins to control levels (Fig. 11A and B respectively).

Discussion

The goal of this study was to determine if daily treatment with Bendavia, a novel mitochondria-targeting peptide, would improve cardiac bioenergetics and result in a better energy supply-demand matching in diabetic hearts. The major findings of this study are: First, treatment with Bendavia improved the ability of diabetic hearts to adjust quickly and efficiently to increased cardiac workload. Second, following treatment with Bendavia, mitochondrial dysfunction was ameliorated as manifested by a better ability to uptake Ca, and an enhanced respiratory capacity. Third, a reductive shift in redox state of diabetic heart mitochondria normalizes mitochondrial Ca. Finally, our data show that the effects of Bendavia are evident at the molecular and functional levels of mitochondrial Ca handling proteins as well as the oxidative phosphorylation machinery.

Cardiac mitochondria from diabetic patients and diabetic animals have a reduced calcium uptake capacity, although the underlying reasons are not understood. Data presented herein implicate the expression of MCU and MCU-regulating proteins as contributors to lower mitochondrial calcium influx. Furthermore, our results show that by targeting the mitochondria we can improve mitochondrial Ca handling, defective cardiac bioenergetics, and the ability of the diabetic heart to increase its myocardial oxygen consumption and its bioenergetics to adjust to changing workloads. To the best of our knowledge, this study is the first to show that MCU, and MCU-regulating proteins are depressed in diabetic hearts, and that their expressions can be re-established to “healthy-state” levels by the mitochondria-targeting peptide Bendavia. Our

findings identify a new therapeutic target which modulation can contribute to ameliorating the energy supply-demand mismatching, a hallmark of diabetic cardiomyopathy, and a major contributing factor to heart failure, and high mortality rates in diabetic patients.

Among the myriad of factors involved in the increased mortality rate associated with diabetic cardiomyopathy, recent evidence suggests that decrements in bioenergetic coordination are centrally involved. While the *healthy* heart is proficient at adjusting its energy supply to demand, the diabetic heart has a very limited capacity of energy matching [17, 18]. A growing body of literature indicates that alterations in mitochondrial function may be responsible for the increased cardiac dysfunction observed in diabetes (reviewed in [19]). Indeed, decreased cardiac mitochondria respiratory capacity has been reported in experimental models of type II diabetes such as db/db [151], and ob/ob mice [152], as well as in the STZ model of type I diabetes [57, 58, 153, 154]. While our results are consistent with this literature, they do shed more light on key components of this mismatching between energy supply and demand in the diabetic heart. Our study is the first to link the decreased mitochondrial Ca uptake, in diabetic hearts, to a decreased expression of the pore forming protein of the mitochondrial Ca uniporter, MCU. Our data are supported by a recently published study using mice lacking MCU [56], where the authors show that in the absence of MCU, mitochondria from skeletal muscle showed slow calcium uptake, and have low levels of Ca in their matrix. This decrease in mitochondrial Ca, manifested by MCU deficient mice, correlated with higher pyruvate dehydrogenase (PDH) phosphorylation and lower PDH activity, and even though it did not affect the baseline whole body oxygen consumption, it did result in reduced performance in situations requiring high-energy expenditure such as the inclined treadmill, or pull-up tests [56]. These new findings confirm that in vivo, mitochondrial calcium uptake is important for muscle activity, particularly when workload is increased. Since excessive mitochondrial Ca uptake leads to permeability transition

pore (PTP) opening, and too little mitochondrial Ca compromises skeletal muscle as well as cardiac muscle bioenergetics, an effective therapeutic approach would be to stimulate MCU activity and effectively inhibit the PTP opening. We have shown that Bendavia does protect against ischemia/reperfusion injury by delaying redox-dependent PTP opening [62], and our data herein suggest that Bendavia ameliorates mitochondrial Ca uptake, under circumstances of increased demand, in a compromised diabetic myocardium.

Our data further show that the expression level of MCUR1, which is essential for mitochondrial Ca uptake and the regulation of cellular metabolism [55], is also decreased in diabetic hearts. The expression level of both MCU and MCUR1 were increased to control levels following treatment with Bendavia, which suggest a new target for Bendavia and maybe a new mechanism of action by which this peptide might ameliorate the heart bioenergetics. Whether this effect is direct by an action on transcription factors or other pathways involved in the regulation of these proteins, or whether it is an indirect effect, resulting from a general decrease of ROS generation, remains to be determined.

In the heart, mitochondrial Ca uptake by MCU only occurs when the cytosolic levels of Ca are high (during systole) which suggest that mitochondrial Ca levels in the mitochondrial matrix are kept low by a highly regulated process. Indeed, Mallilankaraman et al [54] identified MICU1 as the gatekeeper for MCU-mediated Ca uptake. This protein is required to protect mitochondria from Ca overload; however, it does not affect the kinetics of MCU [54]. Diabetic hearts treated or not with Bendavia did not show a significant difference in MICU1 expression level.

It is well documented that diabetic hearts exhibit oxidative stress due to an increased production of reactive oxygen species (ROS) [21, 155] and a decreased ROS buffering capacity [22, 24]. A consequence of this oxidative shift in redox-state is the oxidation of glutathione, the

major thiol buffering system. This has been shown in human diabetic atrium [102], and animal models of diabetes [62, 79]. We have recently confirmed that while Bendavia had no direct scavenging capacity, it does limit the ability of isolated cardiac mitochondria to emit hydrogen peroxide (H_2O_2) [156]. Our results herein show a similar recovery of mitochondrial Ca uptake capability in both cardiac mitochondria from diabetic animals treated with Bendavia, and in cardiac diabetic mitochondria treated with DTT in vitro. These findings suggest that the improved mitochondrial Ca uptake seen in diabetics following treatment with Bendavia might be due, at least in part, to decreased mitochondrial ROS levels. This finding indicates that MCU activity is redox dependent. Further studies aiming to characterize the redox state of MCU in diabetic hearts are warranted to confirm this observation.

High levels of ROS can affect multiple proteins by altering their activity and/or expression levels. Qin et al. [157] reported that post-translational oxidative modification of sarcoplasmic reticulum calcium ATPase (SERCA) led to a decrease in its activity and contributed to the impairment of relaxation. Others also reported the same finding for SERCA in myocardial ischemia/reperfusion injury [158]. We have shown, using the same experimental model of diabetes as the current, that increased propensity to PTP opening is associated with increased oxidative post-translational modification of the adenine nucleotide translocase [62]. So, therapeutic strategies that decrease oxidant levels and/or protect target proteins, such as MCU, from oxidation might be of value to preserve the cardiac function in diabetic patients.

The fact that Bendavia improves Ca uptake in cardiac diabetic mitochondria, and results in the recovery of MCU expression levels suggests that this mitochondria-targeting peptide might regulate both the activity and the expression of MCU. This double action might be indirectly mediated by a reduction in ROS levels or, it might be due to a yet unknown mechanism of action of this peptide.

A direct consequence of an improved mitochondrial Ca uptake is a better capacity to generate ATP to match the cardiac myocyte needs. Indeed, it is well recognized that mitochondrial Ca fluxes are integral to ATP supply and demand matching (reviewed in [10]). Ca serves as a key activator of several Krebs cycle dehydrogenases such as PDH, glycerol 3-phosphate dehydrogenase, NADH-linked isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, as well as the ATP-synthase [51, 159-161]. Consistent with the relationship between mitochondrial Ca and oxidative phosphorylation capacity, our results show an improvement of mitochondrial respiratory capacity with different substrates under phosphorylating state, and this is concomitant with an increase in the expression level of complex I and V of the mitochondrial respiratory chain. This suggest that treatment with Bendavia results in an improvement of the cardiac mitochondrial function that might be explained by a dual improvement of the expression and activity of mitochondrial proteins involved in Ca uptake and oxidative phosphorylation. This finding is also supported by the correlation between decreased PDH activity and the lack of MCU shown by Pan et al in MCU deficient mice [56].

One limitation to our study is the use of type I diabetes animal model (streptozotocin-induced diabetes) instead of a type II diabetes model. The most common form of diabetes is diabetes mellitus type II, but both types of diabetes show similarities as reported in clinical and experimental studies. Just like cardiac mitochondria from animals with type II diabetes, mitochondria from hearts of type 1 diabetic animals exhibit reduced oxidative phosphorylation capacity, a lower ATP synthase activity, [57], a decreased mitochondrial calcium uptake [58, 62, 69], and an increased oxidative stress burden [24].

Cardiovascular disease remains the leading cause of mortality and morbidity in individuals with diabetes. This cardiovascular-associated mortality is believed to be mainly a

consequence of coronary artery disease. However, compelling epidemiological and clinical data indicate that diabetes increases the risk for cardiac dysfunction and heart failure independently of other risk factors such as coronary disease and hypertension, as firstly reported by Rubler in 1972 [162]. Furthermore, mounting data point to mitochondrial dysfunction as being a major early contributor to diabetic cardiomyopathy. It is then, critical to develop therapeutic strategies targeting mitochondria to correct, and maybe prevent early defects in cardiac mitochondrial function in order to prevent the myocardial bioenergetic defects from leading to heart failure.

In conclusion, our study aimed to understanding how Bendavia improves bioenergetics in diabetic hearts. This study provides novel evidence that this mitochondria-targeted peptide is a plausible and promising candidate for the reduction of diabetic cardiomyopathy in its early stages, by correcting mitochondrial dysfunction at different levels.

Table 4

Morphological, metabolic and hemodynamic data from control and diabetic rats treated with Bendavia (1.5 mg Kg^{-1} day^{-1}) or vehicle (saline)

Parameters	**Control-Saline (n=6)**	**Control-Bendavia (n=6)**	**STZ-Saline (n=13)**	**STZ-Bendavia (n=13)**
Body weight (g)	361 ± 12	372 ± 6	225 ± 6 *#	250 ± 14 *#
Heart weight (g)	1.44 ± 0.13	1.42 ± 0.07	1.08 ± 0.05 *#	1.06 ± 0.06 *#
LV weight (g)	0.81 ± 0.07	0.87 ± 0.04	0.61 ± 0.03 *#	0.62 ± 0.04 *#
RV weight (g)	0.20 ± 0.01	0.21 ± 0.01	0.14 ± 0.01 *#	0.15 ± 0.01 *#
HW/TL	37 ± 4	40 ± 2	33 ± 2	31 ± 2
HW/BW	3.60 ± 0. 31	3.82 ± 0.23	4.81 ± 0.19 *#	4.28 ± 0.23
Fasting blood glucose (mg/dl)	112 ± 4	144 ± 10	488 ± 24 *#	467 ± 44 *#
MVO_2 max (µmole/min*g dry HW)	69 ± 6	65 ± 2	63 ± 6	85 ± 6
LVDP (mmHg)	143 ± 11	118 ± 9	122 ± 12	95 ± 14

Results are expressed as means ± SEM (n= 6-13 rats per group).
STZ, streptozotocin; LV, left ventricle; RV, right ventricle; HW, heart weight; TL, tibia length ; BW, body weight, MVO_2max, maximal myocardial oxygen consumption following injection of the uncoupler; FCCP, LVDP, left ventricular developed pressure at baseline. Statistical significance: * *P<0.05 vs.* Control-Saline group # *P<0.05 vs.* Control-Bendavia group.

Figure 8

A.

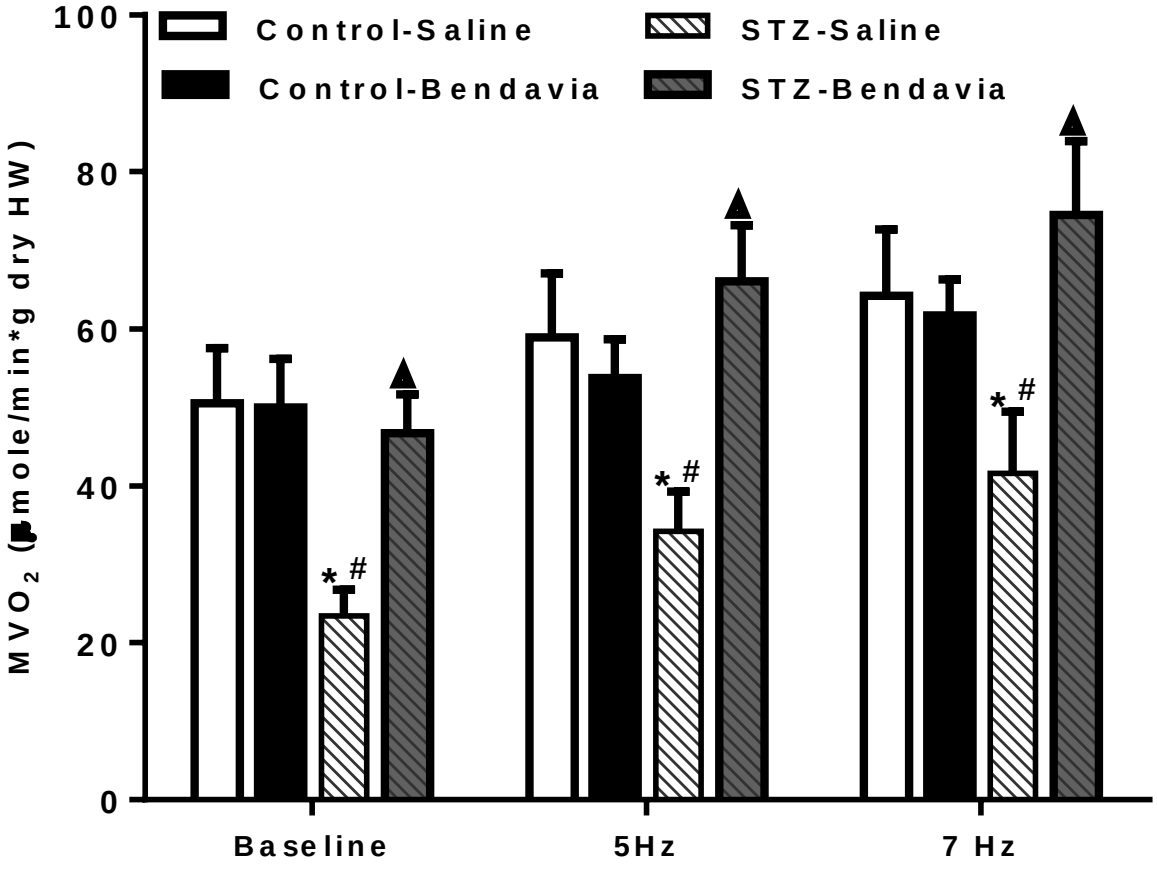

Control-Saline
STZ-Saline
Control-Bendavia
STZ-Bendavia
100
80
60
40
20
0
MVO2 (µmole/min*g dry HW)
*
#
Baseline
5Hz
7 Hz

Figure 8 (cont'd)

B.

Figure 8 (cont'd)

C.

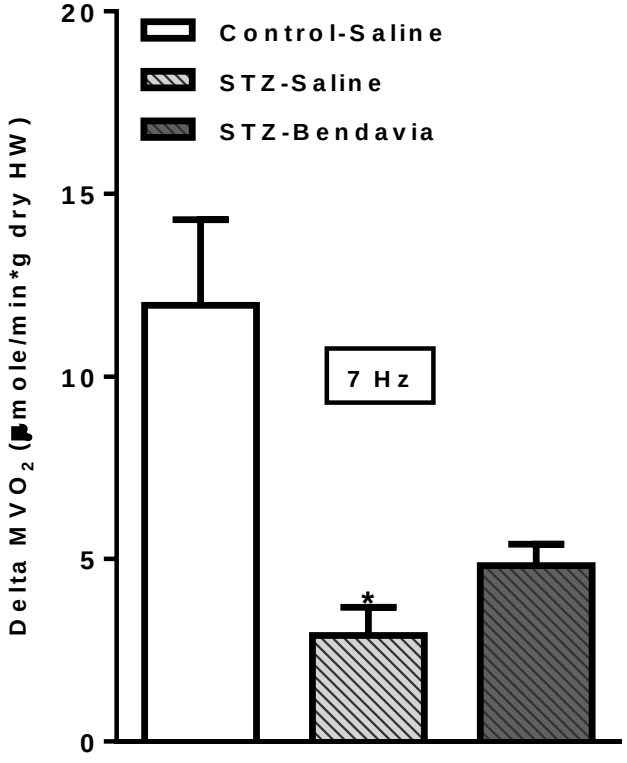


Figure 8 (cont'd)

D.

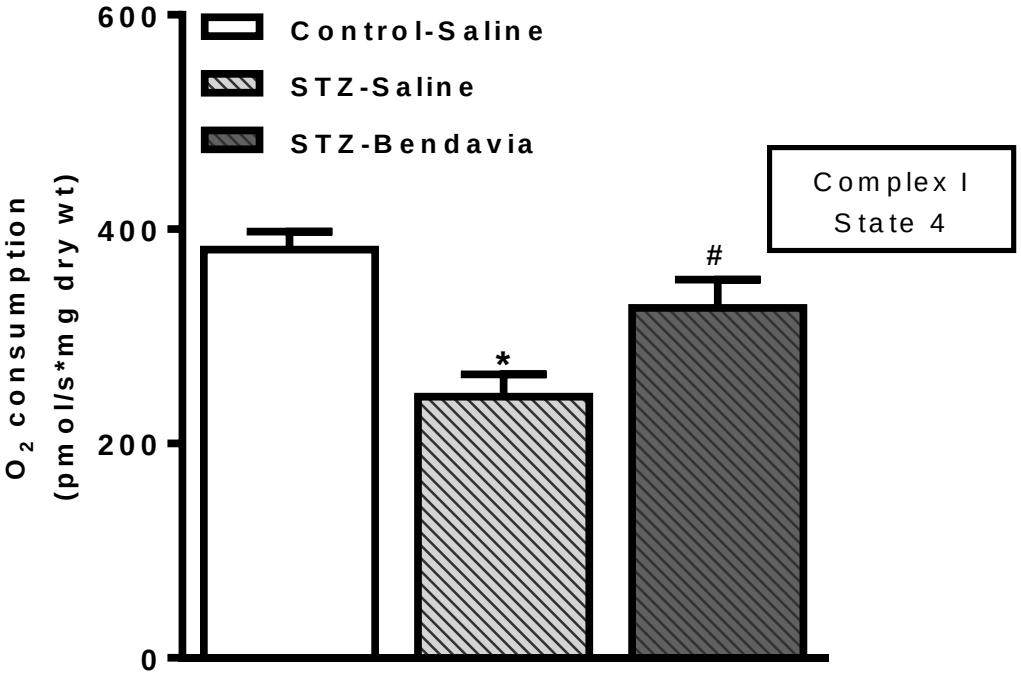


Figure 8 (cont'd)

E.

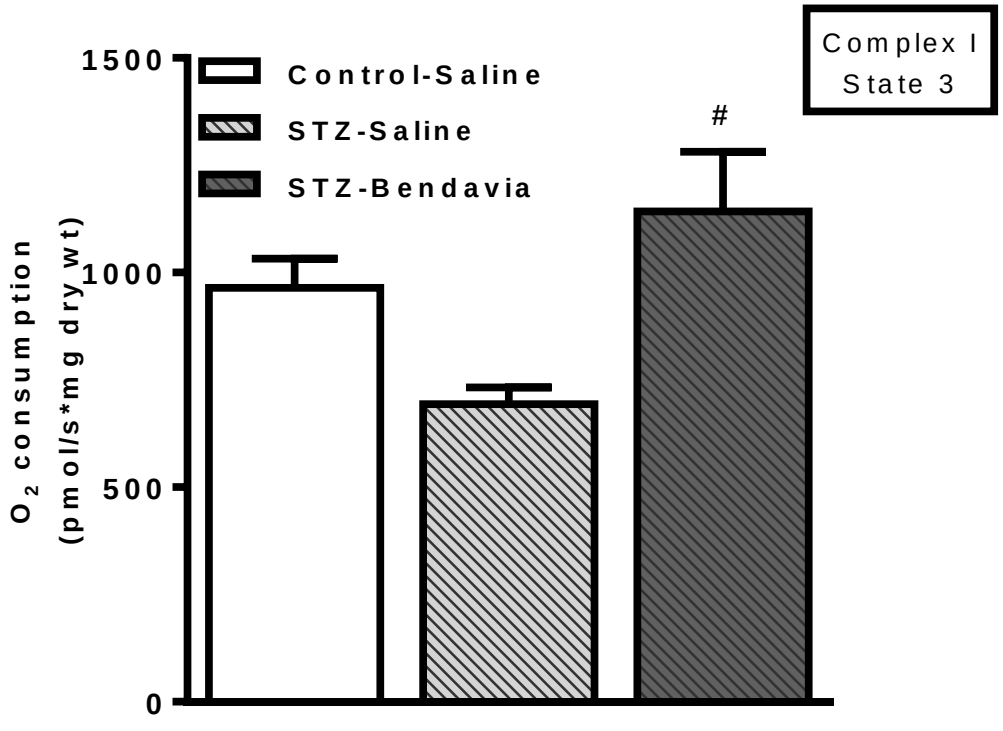


Figure 8 (cont'd)

F.

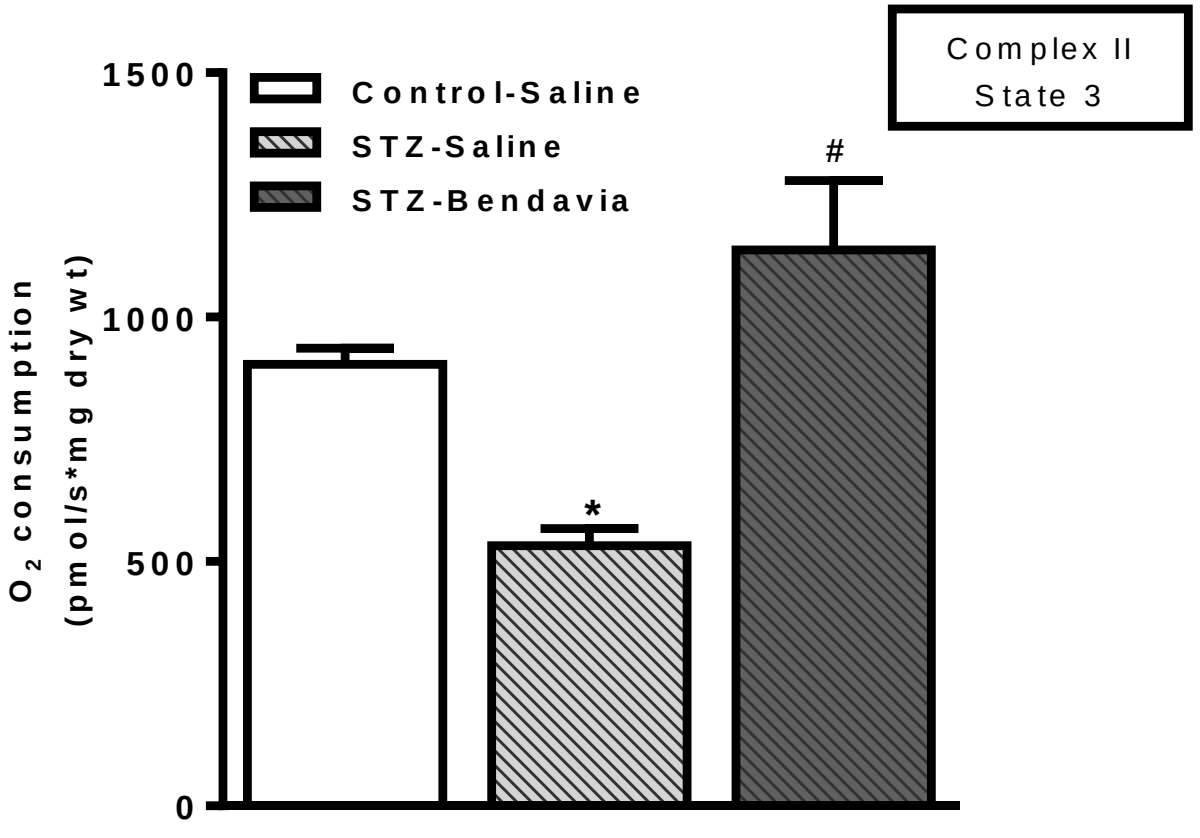
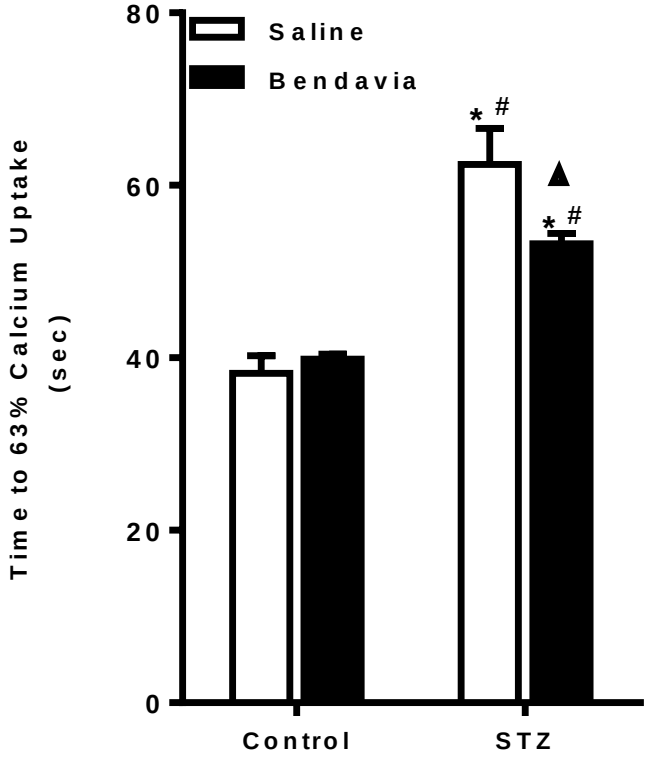


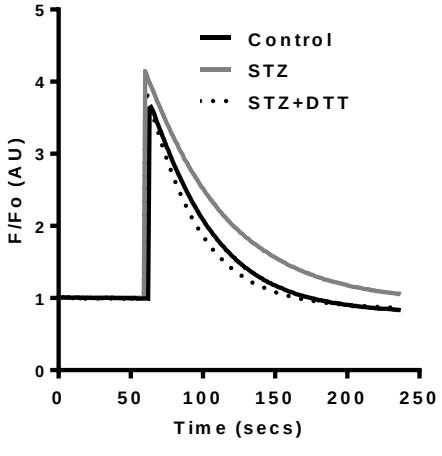
Figure 8: Diabetic animals display a decreased cardiac and mitochondrial oxygen consumption that were restored following treatment with Bendavia. Myocardial oxygen consumption (MVO_2) in control and diabetic rats treated with vehicle or Bendavia (1.5 mg/Kg for 4 days) was measured in perfused hearts at baseline, and after increasing cardiac workload by pacing the hearts continuously at 5Hz then 7 Hz for 3 minutes each time (**A**). The ability of control and diabetic hearts to adjust their oxygen consumption to the increased workload was assessed in isolated perfused hearts by measuring the change in myocardial oxygen consumption (Delta MVO_2, which corresponds to change in MVO_2 from baseline level to level immediately following pacing) with changing workloads at 5Hz (**B**) and 7 Hz (**C**). Complex I-supported-respiration was measured in skinned cardiac fibers from control and diabetic animals using glutamate and malate as substrates, in the absence (**D**), and the presence (**E**) of ADP. Complex II supported-respiration was assessed in the presence of rotenone and ADP using succinate as substrate (**F).** Data are means ± SEM of 6-8. Statistical significance: **P*<0.05 vs. Control-saline group; #*P*<0.05 vs. Control-Bendavia group; Δ*P*<0.05 vs. STZ-saline group.

Figure 9

A.


Saline
Bendavia
Time to 63% Calcium Uptake (sec)
80
60
40
20
0
* #
* #
Control
STZ

B.


Control
STZ
STZ+DTT
F/Fo (AU)
5
4
3
2
1
0
0
50
100
150
200
250
Time (secs)

C

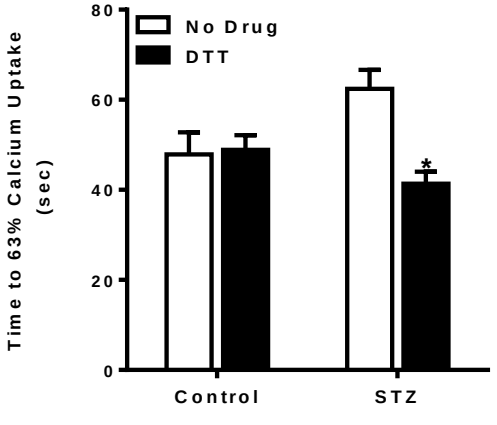

No Drug
DTT
Time to 63% Calcium Uptake (sec)
80
60
40
20
0
*
Control
STZ

Figure 9 (cont'd)

D

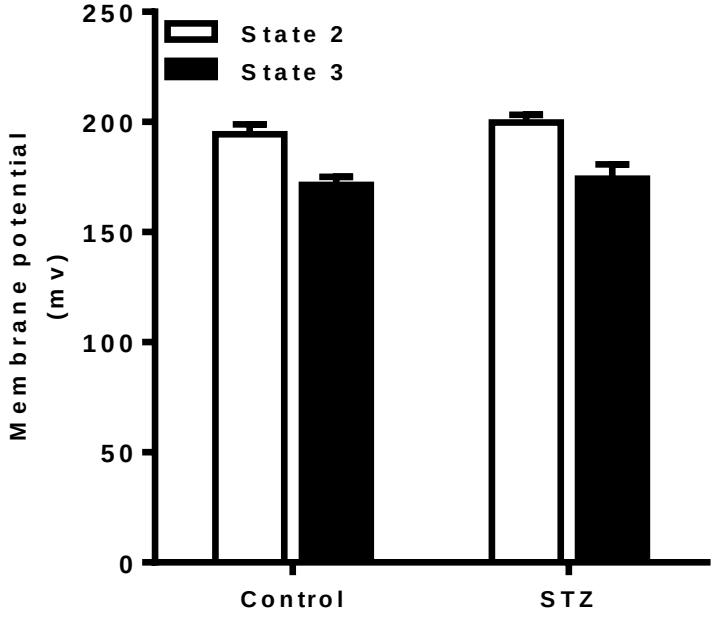


Figure 9: Diabetic animals display a decreased cardiac mitochondrial function that was improved following treatment with Bendavia**.** All experiments were conducted in control and diabetic rats treated with vehicle or Bendavia (1.5 mg/Kg for 4 days). Mitochondrial Ca uptake was measured in isolated cardiac mitochondria using a Ca-sensing probe (calcium green-5N salt), and the time it took mitochondria to accumulate 63% of total Ca following one pulse of $CaCl_2$ was determined in non-pretreated mitochondria (**A**) or DTT-pretreated mitochondria (**C**). Representative trace of Ca uptake is displayed in **B**. Mitochondrial membrane potential was measured in isolated cardiac mitochondria using an electrode sensitive to TPP under state 2 (no ADP) and state 3 (2.5 mM APD) respirations (**D**). Data are means ± SEM of 6-8. Statistical significance: **P*<0.05 vs. Control-saline group; #*P*<0.05 vs. Control-Bendavia group; Δ*P*<0.05 vs. STZ-saline group.

Figure 10

A.

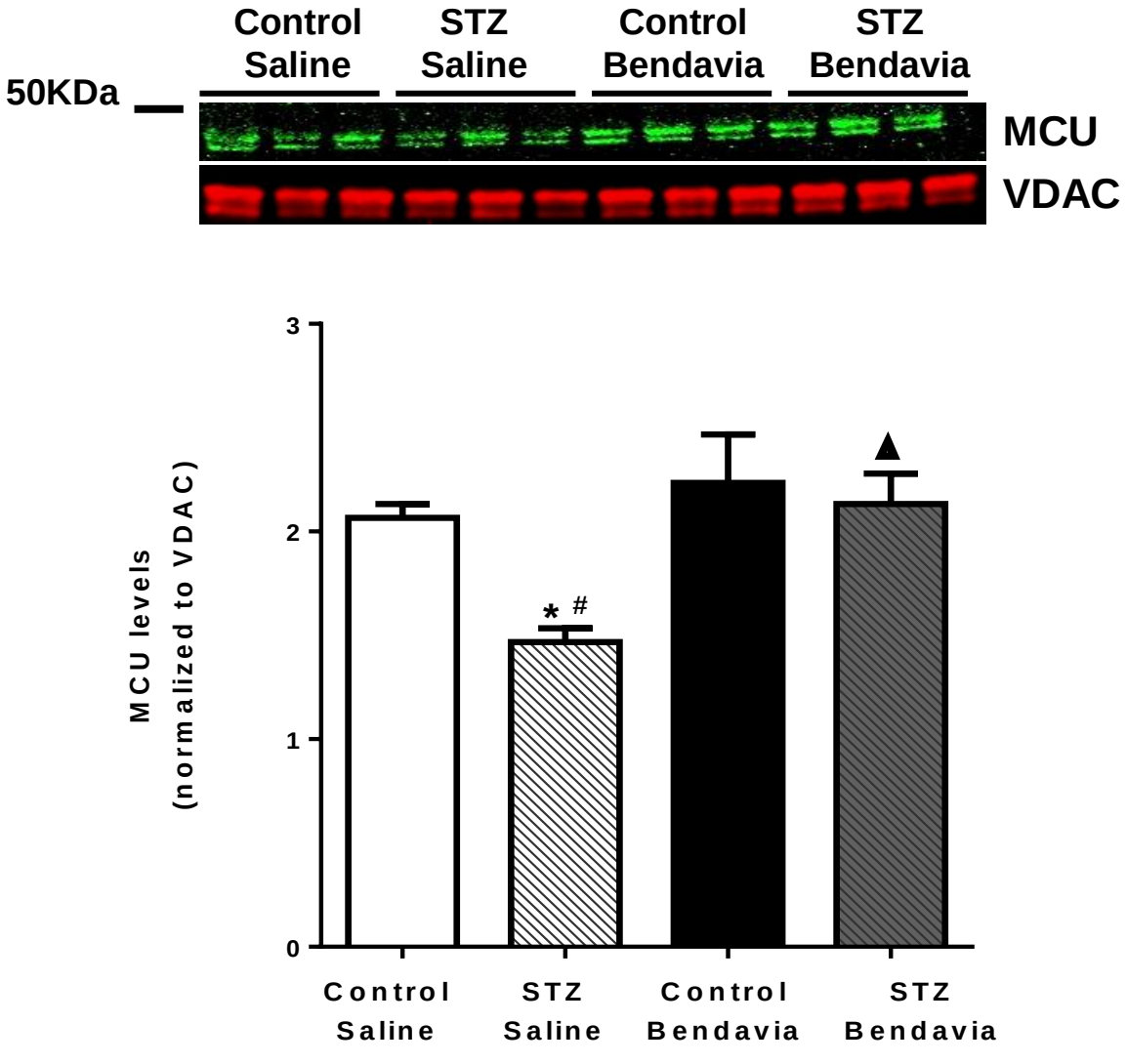

Control Saline
STZ Saline
Control Bendavia
STZ Bendavia
50KDa
MCU
VDAC
MCU levels (normalized to VDAC)
3
2
1
0
* #
Control Saline
STZ Saline
Control Bendavia
STZ Bendavia

Figure 10 (cont'd)

B.

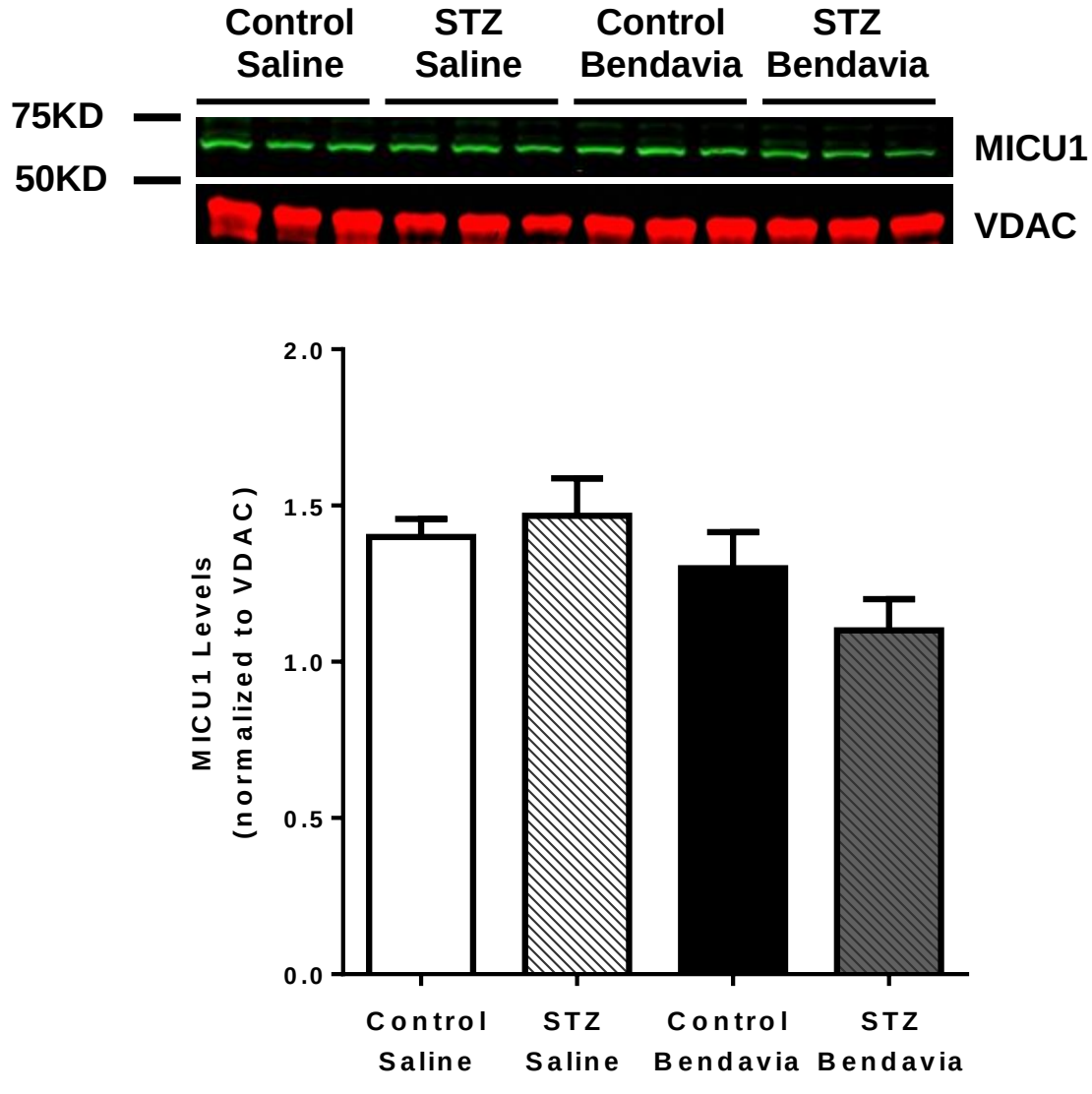


Figure 10 (cont'd)

C.

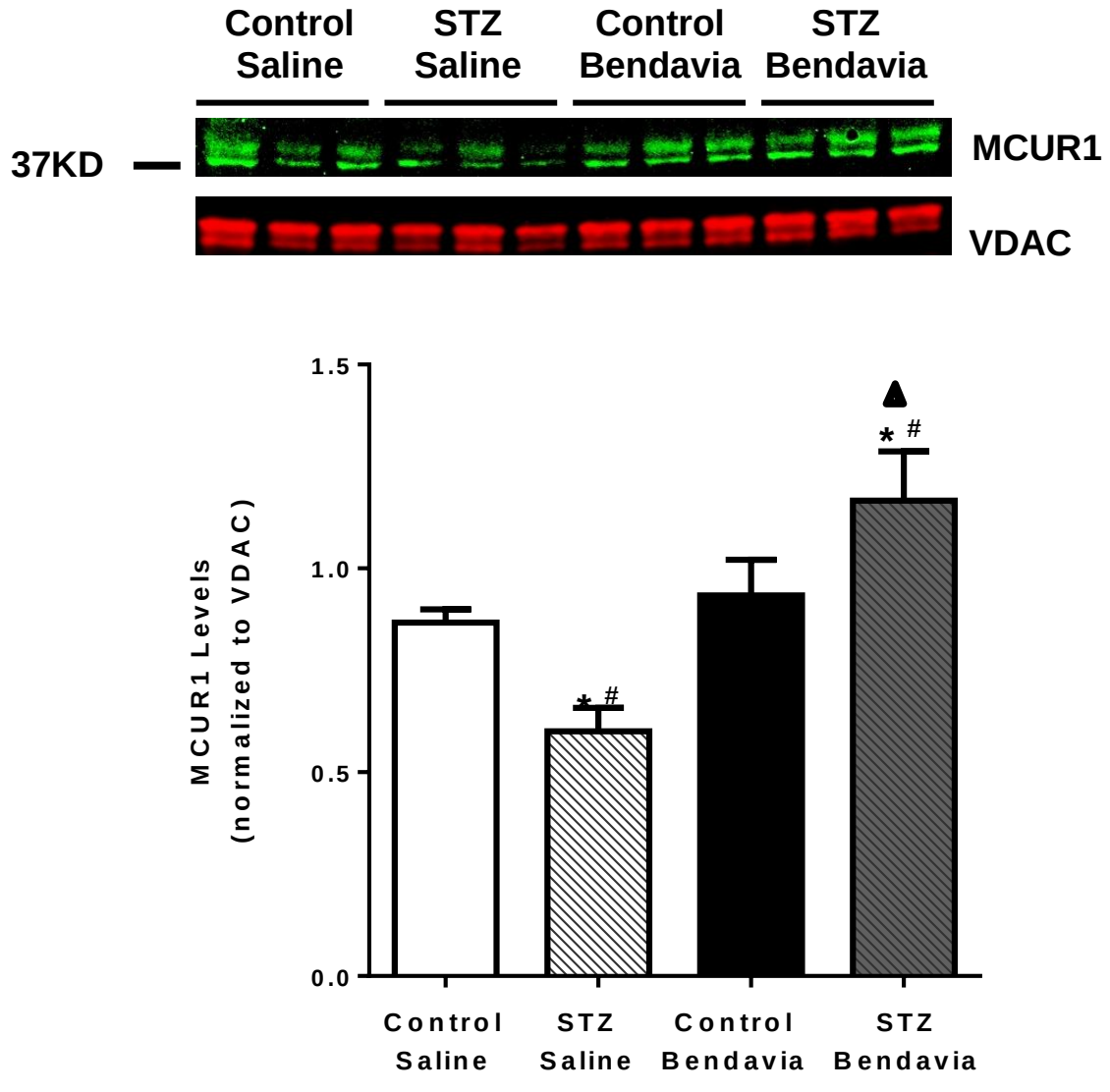


Figure 10: Diabetic animals display a decreased expression of mitochondrial Ca handling proteins which was improved following treatment with Bendavia. Western blots of mitochondrial Ca handling proteins as assessed in cardiac mitochondrial extracts. **A**: MCU, **B**: MICU1, and **C**: MCUR1. Quantitative analyses are reported in the histograms, and they represent the expression level (band densitometry) of each protein normalized to the mitochondrial protein VDAC. Data

are means ± SEM of 3. Statistical significance: *P<0.05 vs. Control-saline group; #P<0.05 vs. Control-Bendavia group; ΔP<0.05 vs. STZ-saline group.

Figure 11

A.

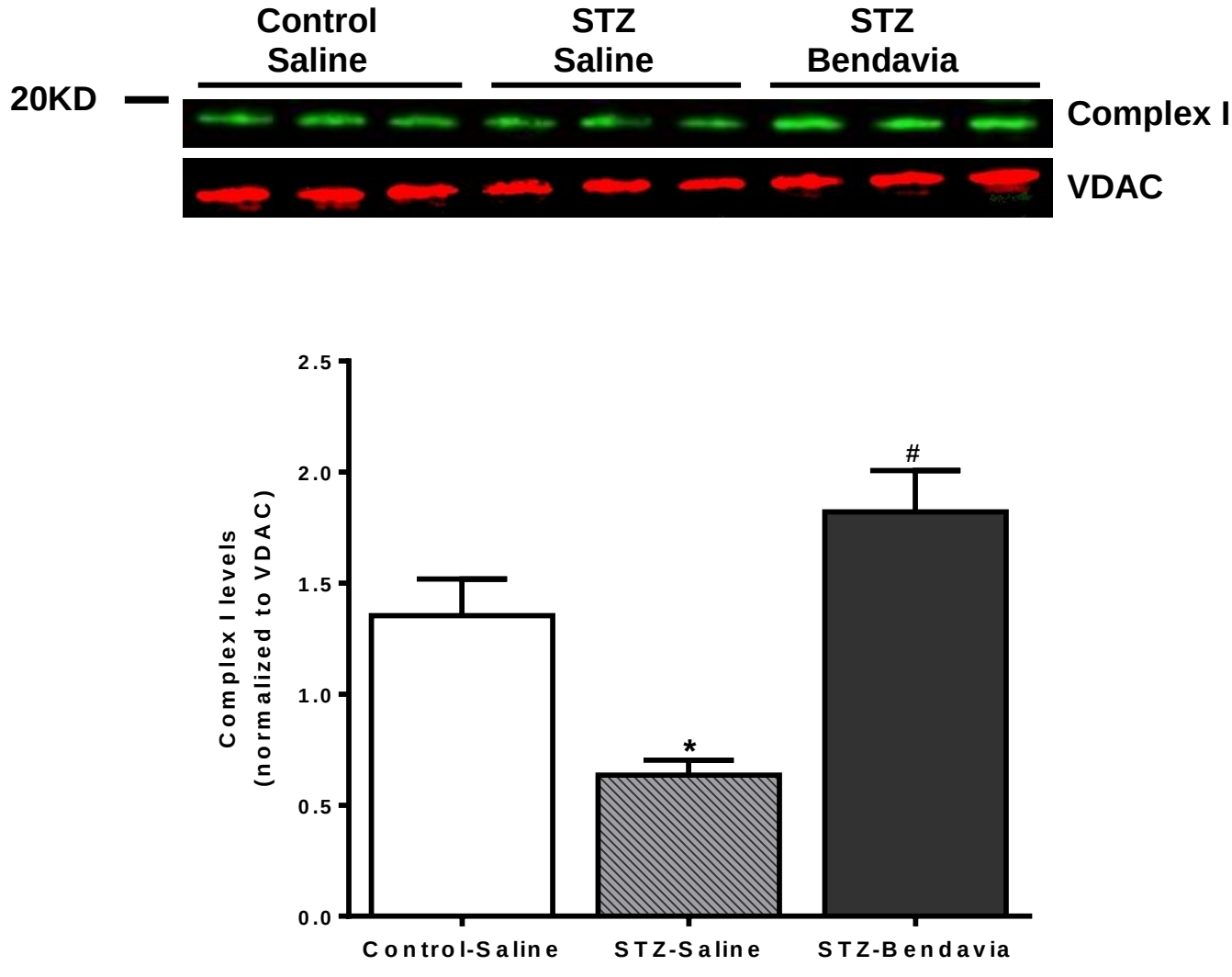

Control
Saline
STZ
Saline
STZ
Bendavia
20KD
Complex I
VDAC
Complex I levels
(normalized to VDAC)
2.5
2.0
1.5
1.0
0.5
0.0
#
*
Control-Saline
STZ-Saline
STZ-Bendavia

Figure 11 (cont'd)

B.

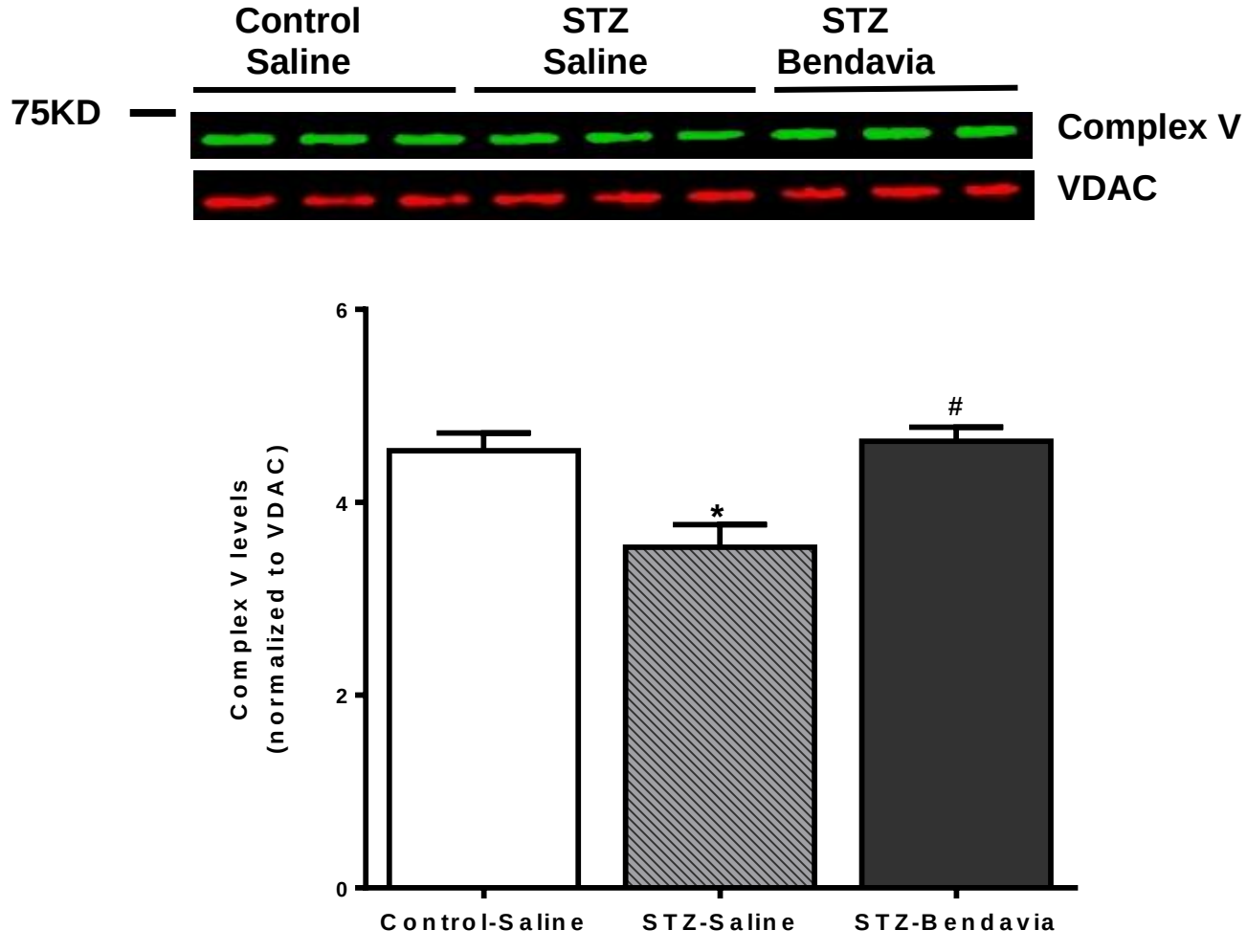


Figure 11: Diabetic animals display a decreased expression of oxidative phosphorylation proteins which was improved following treatment with Bendavia. Western blots of mitochondrial respiratory complexes as assessed in cardiac mitochondrial extracts. **A**: Complex I and **B**: Complex V (F1/Fo-ATPase). Quantitative analyses are reported in the histograms, and they represent the expression level (band densitometry) of each protein normalized to the mitochondrial protein VDAC. Data are means ± SEM of 3. Statistical significance: **P*<0.05 vs. Control-saline group; #*P*<0.05 vs. STZ-saline group.

Chapter 4: Macromolecular organization of the mitochondrial respiratory complexes following myocardial ischemia reperfusion injury, and its impact on mitochondrial function

Introduction

Dysfunction of the mitochondrial respiratory complexes (MRCs) are highly associated with the decline in mitochondrial bioenergetics, and this decline is a major contributor to myocardial injury resulting from ischemia/reperfusion (IRI) [8, 163-166].

The direct consequence of IR-induced structural alterations of the MRCs is inefficient electron transfer. The latter results in secondary electron leak leading to increased ROS generation [166-168]. Furthermore, IRI is also associated with a decrease in the ROS buffering capacity. For instance, the myocardial glutathione system, which is a major cardiac antioxidant [101], shows a significant decline in its ROS buffering ability following IRI as reflected by a decrease in the glutathione redox couple (GSH/GSSG) as we [62, 84], and others [169] have shown in rodent hearts. Thus, the increase in ROS generation coupled with reduced antioxidant capacity leads to a net accumulation of ROS. These highly reactive molecules can interact with the mitochondrial proteins and lipids and initiate their oxidative modification. Indeed, oxidation of multiple subunits of complex I [169, 170], and complex II [169, 171] was reported after cardiac IRI. When MRC proteins are the target of this oxidative modification, they show structural and functional changes leading to an overall decline in mitochondrial bioenergetics [172, 173].

Recent evidence, based on the separation of MRCs by BN-PAGE, suggests that they can be organized in supramolecular assemblies called supercomplexes or respirasomes [31, 36-38]. This organization has functional features because higher rates of respiration are achieved when

the MRCs are agglomerated into supercomplexes. The reason for this higher mitochondrial efficiency is believed to be granted by the optimal vicinity between different redox partners resulting in substrate channeling, a more efficient electron transfer, and less electron loss (reviewed in [45]). The supercomplexes are mostly the result of the association between complexes I, III, and IV at different stoichiometries depending on the organism and the organ studied.

The levels of supercomplexes were reported to be decreased in a canine model of heart failure [174]. This decrease in the amount of respirasomes was accompanied by a significant loss of efficient electron transfer in subsarcolemmal and intrafibrillar cardiac mitochondria, indicating an association between the supramolecular organization of the MRCs and the mitochondrial function. In addition, the integrity as well as the stochiometry of different MRCs is essential to the formation and stability of the supercomplexes [29]. Indeed, in Barth syndrome and Leigh syndrome where complex I and complex III structures are altered, the assembly of supercomplexes is also altered and is associated with a decreased mitochondrial function.

In chapters 2 and 3, we used well established experimental models of diabetes and IRI in order to gain insight into the mechanisms behind the aberrant cardiac bioenergetics associated with these two disease states. We showed significant molecular changes of multiple mitochondrial proteins (mitochondrial Ca-handling proteins and multiple respiratory complexes). These molecular changes were associated with mitochondrial and cardiac dysfunctions, both of which were improved in presence of the protective inner mitochondrial membrane-targeting peptide, Bendavia. So, in this chapter we investigated the impact of the MRCs molecular organization on cardiac bioenergetics following IRI. We assessed the correlation between the respiratory rate and the profile as well as the content of mitochondrial supercomplexes (supra-molecular organization of MRCs). We utilized Bendavia [2] for its established protective effects

against IRI [175-177] and looked at its effect on the content and integrity of mitochondrial supercomplexes in the post-ischemic heart.

Methods

Animals

Male Sprague-Dawley rats (aged 2-3 months) were used in the study. All procedures received prior approval from the East Carolina University Institutional Animal Care and Use Committee. Animals were housed in a temperature and light-controlled environment and received food and water ad libitum. Animals received an i.p. injection of a ketamine/xylazine (90mg/kg/10mg/kg, respectively), and hearts were excised after the diminution of animal reflexes, via midline thoracotomy and placed in ice-cold saline. Excised hearts were perfused on our modified Langendorff apparatus per our established protocols [87, 90]. Hearts were exposed to 20/120 minutes of global ischemia/reperfusion. For the Bendavia treatment, hearts received 1nM Bendavia beginning at the onset of reperfusion, which is a well-established cardioprotective paradigm in our models [175, 177]. At the end of reperfusion, hearts were split into the experimental groups described below.

Separation of native mitochondrial respiratory chain complexes by BN-PAGE

Separation of mitochondrial supercomplexes was performed using BN-PAGE according to methods described by Schägger et al. [36]. Mitochondrial membranes were solubilized using either digitonin (digitonin: mitochondrial protein ratio was 8:1 (w/w)) or n-dodecyl-β-D maltoside (DDM:protein ratio of 2:1) in a buffer containing 6N HCl, 50 mM NaCl, 0.001% Ponceau S, and 50 mM Bis-Tris, pH 7.2. After solubilization, the samples were centrifuged for 30 min at 20, 000 x g, and protein content of the supernatant was determined using bicinchoninic acid (BCA protein assay reagent, Thermo Scientific, Rockford, IL, USA). Coomassie G-250 was

added to each sample in a ratio detergent-to-dye of 8:1 (w/w), and 35 µg total proteins from each sample were separated on a Native PAGE 3-12% Bis-Tris (Life Technologies, Carlsbad, CA, USA) at 4°C. Running buffer was supplemented with 0.02% Coomassie G-250 to allow visualization of protein bands. The stained bands were scanned using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NB), and the densitometric analysis was done using Image J software (National Institutes of Health).

High-resolution respirometry.

Following the I/R injury, the left ventricle was dissected and placed on a petri dish containing ice-cold buffer X containing in (mM): 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 20 Taurine, 5.7 ATP, 14.3 Phosphocreatine, 6.56 $MgCl_2$-$6H_2O$ and 50 MES; pH 7.1. All fat and connective tissue was removed under a dissecting microscope, and small cardiac fiber bundles (5-7 mg of wet weight) were prepared. Fiber bundles were permeabilized using 50µg/ml saponin at 4°C for 20 min. Permeabilized fiber bundles were then washed, 3 times for 5 min each, in ice-cold buffer Z containing in (mM): 110 K-MES, 35 KCL, 1 EGTA, 5 K_2HPO_4, 3 $MgCl_2$-$6H_2O$, and 5 mg/ml BSA; pH 7.4. The fiber bundles were kept at 4°C in buffer Z until analysis.

All experiments were conducted at 37°C in presence of 20 µM blebbistatin to prevent contraction. We used the Oroboros system (Oroboros Instruments, Innsbruck, Austria) to measure O_2 consumption per our established protocols [146]. Buffer Z was used as respiration medium and O_2 consumption was monitored during a substrate-inhibitor titration oxygraphic protocol in order to obtain a step-by-step analysis of various components of the mitochondrial respiratory chain as described by Kuznestov et al. [147]. The addition of substrates and inhibitors was done in the following sequence: glutamate/malate (10mM/5mM), ADP (2 mM), rotenone (0.5 µM), succinate (10 mM), antimycin A (5 µM), N,N,N',N'-tetramethyl-p-phenylenediamine

(TMPD)/ascorbate (0.5mM/2mM), cytochrome C (10 μM), and FCCP (1 μM). At the end of each experiment the fiber bundles used were washed with distilled water and lyophilized in a freeze-dryer (Labconco, Kansas city, MO) for 3 hours and then weighed on a microsclae (Mettler-Toledo XS3DU). O_2 consumption is expressed in pmol/min*mg dry wt. FCCP data were excluded from 3 fibers because they were statistical outliers, with values > 2 standard deviations away from the group mean.

Statistical analyses.

All data were analyzed using and GraphPad Prism and are presented as mean ± SEM. We ensured the data were distributed normally, which allowed for parametric analyses. Statistical analyses were conducted using a one-way ANOVA, with p values < 0.05 considered significant. To conduct statistical analyses with fluorescence studies, the percent change were calculated based on the maximal fluorescence value for fluorescence emission.

Results

Blue-native PAGE studies.

Blue-native gels are presented in Fig. 12. The density of mitochondrial supercomplexes was significantly lower following ischemia-reperfusion, and post-ischemic administration of Bendavia significantly improved the formation of respiratory supercomplexes (Fig. 12A; $P<0.05$). The expression of native complex I was higher in the ischemia-reperfusion group, and normalized with Bendavia perfusion (Fig. 12B and C). Native Complex V expression was significantly depressed with ischemia-reperfusion, and brought back to control levels with Bendavia treatment (Fig. 12B and D). There were no differences in endogenous complex III_2 expression among groups in the study (Fig. 12E). Interestingly, we observed the presence of 'breakdown' products in the ischemia-reperfusion group, with the most prominent molecular

weights being between 400-500kDa. Post-ischemic treatment with Bendavia significantly attenuated the presence of these 'degradation' bands (Fig. 12F; $P<0.05$).

High-resolution respirometry in permeabilized ventricular fibers.

Respiratory control ratios (during glutamate/malate-stimulated respiration) were 3.6 ± 0.2 in control fibers. As expected, the respiratory control ratio decreased after ischemia-reperfusion (1.9 ± 0.1; $P<0.05$ versus control), and this drop was blunted in the ischemia-reperfusion + Bendavia group (2.5 ± 0.1; $P<0.05$ versus both ischemia-reperfusion and control). We employed a substrate-inhibitor titration protocol to ascertain how each component of the electron transport chain was influenced by ischemia-reperfusion. Respirometry data are presented in Fig. 13. States 3 and 4 respiration were significantly lower in the ischemia-reperfusion group, and this decline was partially reversed with Bendavia across groups, regardless of whether the mitochondria were respiring on glutamate/malate or succinate/rotenone (Fig. 13A and B). Both complex IV-dependent and FCCP-induced respiration were decreased following ischemia-reperfusion, and Bendavia had no statistically significant effect in these studies (Fig. 13C and D).

Discussion

The main findings of our study are that IRI causes a significant decrease in mitochondria bioenergetics, considerable molecular changes of the individual respiratory complexes as well as their organization into supercomplexes and that the application of Bendavia at reperfusion is able to protect the mitochondria of ischemic hearts against all these structural and functional decays.

Specifically, our data show a significant decrease in the content of supercomplexes, a loss of complex I integrity (appearance of degradation bands), and a significant decrease in the levels of complex V. The loss of complex I integrity is indicated by a combined decrease in its molecular weight, the appearance of small molecular weight bands, and a reduced efficiency in

electron transfer; all of which suggest complex I instability and possible degradation. This degradation is consistent with data in the literature showing an association between a decline in supercomplex content and the loss of both the integrity (loss of subunits) and the content of complex I. Since both normal expression of each individual respiratory complex, and sufficient quantities of the assembled supercomplexes are crucial for the mitochondrial function [178], our study identifies mitochondria-targeting peptide Bendavia as a suitable pharmacological approach to preserving mitochondrial bioenergetics by preserving the MRCs under individual and supercomplex forms.

Following IRI, complex I is described to be in its deactivated form (there are 2 forms; A, active and D, deactivated) [166]. When in its D form, complex I can receive electrons from NADH but can't deliver them to ubiquinon, so electrons leak generating ROS instead [166]. In the latter study, complex I oxidative capacity and its enzymatic activity were decreased and ROS generation was increased. Similar findings were reported by multiple studies showing a decline in complex I catalytic activity following IRI [166, 179-181]. In addition, pharmacological interventions aiming to inhibit complex I, during early reperfusion, protected mitochondria and heart tissue from ischemic injury and enhanced cardiac functional recovery [182-185]. In the study by Lesnefsky et al. (2004), the improved cardiac function was associated with a decrease in ROS generation by complex I, a prevention of cardiolipin loss, and an improved oxidative capacity of cytochrome c oxidase [182]. Interestingly, the assembly of MRCs, into supercomplexes stabilizes CI [186] and minimizes its ability to generate ROS [30]. Taken together these data suggest that following IRI, complex I structure and activity are altered leading to an overall decrease in its oxidative capacity and an increase in its ability to generate ROS, and that these changes might be related to the inability of complex I to integrate the supercomplex assembly.

Our data are consistent with the molecular changes of complex I, and its decreased oxidative capacity reported in the literature in the context of myocardial IRI. In addition, our data show that under native conditions, complex I of control hearts is mostly incorporated into supercomplexes, and this is coupled with a superior mitochondrial function. Again, this is consistent with the proposed role of supercomplex organization as it confers stability and efficiency to complex I (reviewed [45]). We also show that the inner mitochondrial membrane-targeting peptide Bendavia is able to protect complex I from these molecular and functional alterations since its integrity and oxidative capacity are preserved in the presence of Bendavia.

A number of human genetic disorders, caused by a single mutation, lead to deficiencies in more than one respiratory complex [187-189]. Such defects stem from the interdependence between the different MRCs and from the fact that their stability is dependent on their assembly into supercomplexes. In fact, the structural integrity of individual MRCs and their number in relation to each other (specific stoichiometry) are essential to their assembly into supercomplexes, which in turn is required for the assembly, stability and catalytic efficiency of individual respiratory complexes [38, 188]. For instance, genetic mutations leading to defects in the assembly of complex III are associated with a decrease in the expression level, the activity and the incorporation of complex I into supercomplexes [38]. This relationship was shown in mouse and human-derived cultured cells as well as in muscle biopsies from patients with the same type of mutation. This finding was confirmed by another group while investigating the structural and functional consequences of the loss of complex IV and complex III on the composition and integrity of supercomplexes [188]. Using skeletal muscle biopsies from patients with Leigh syndrome (deficiency of complex IV), and patients with cytochrome b mutations (deficiency of complex III), Schagger and colleagues showed that while the loss of complex IV didn't seem to affect the levels of complex I, that of complex III prevented the formation of

supercmplexes and resulted in a secondary loss of complex I. Similar association between complex IV/complex III and complex I was also shown in bacteria (Paracoccus denitrificans) [190]. Taken together, these results suggest that complex I assembly and activity are dependent on its integration into supercomplexes which in turn is dependent on the integrity of other MRCs such as complex III. This relationship between supercomplexes content and free complexes assembly and integrity could explain the results we see in IRI. We have a decrease in supercomplexes content and signs of degradation of complex I. Indeed the molecular weight of complex I is decreased in IRI group and new bands of lower molecular weight appear. This suggests that when complex I is not integrated into the supercomplex assembly it is either degraded or its structure is modified and this is consistent with the literature.

Further studies are warranted in order to determine the composition of the supercomplexes as well as the degradation bands.

Figure 12

A.

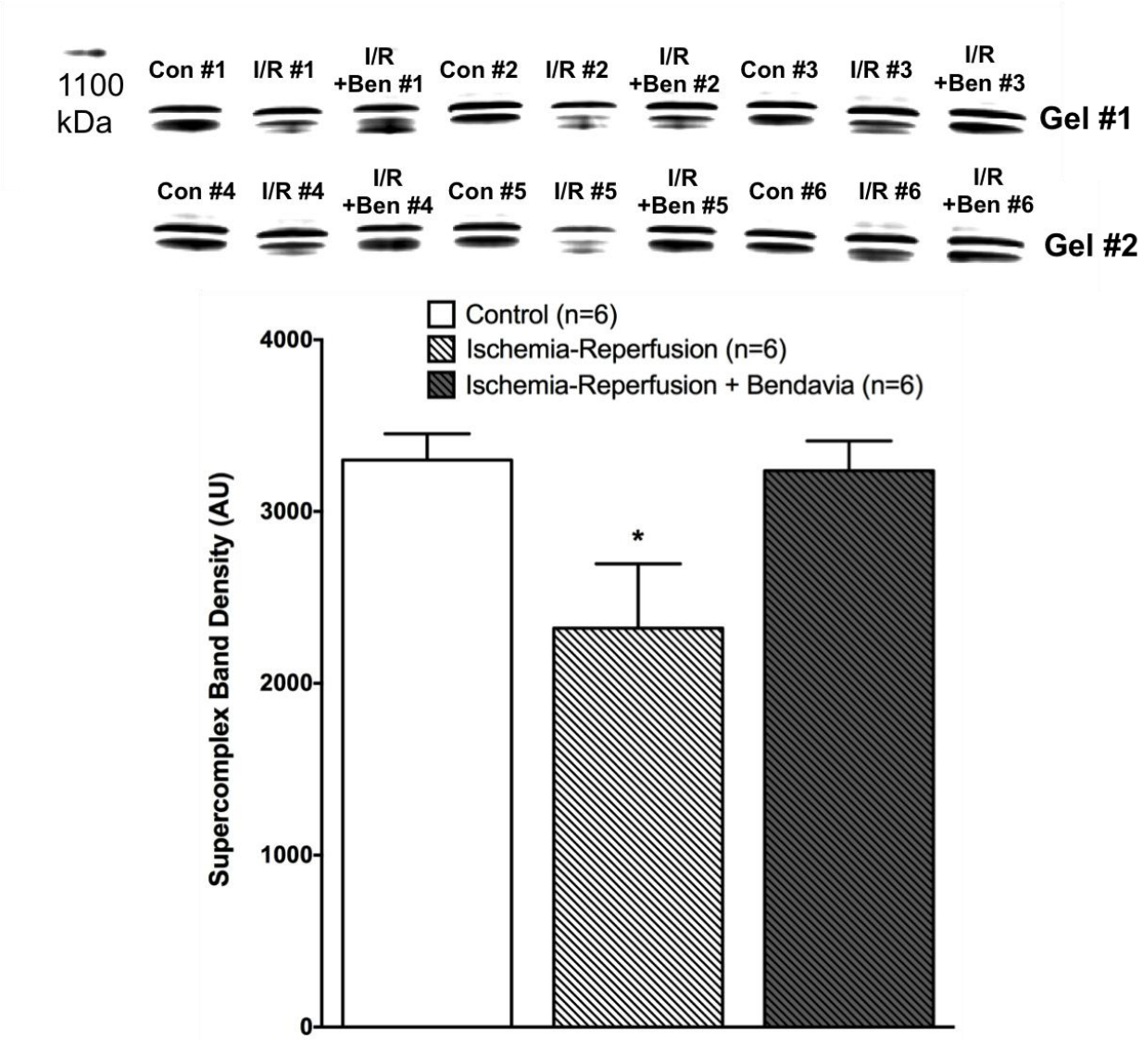
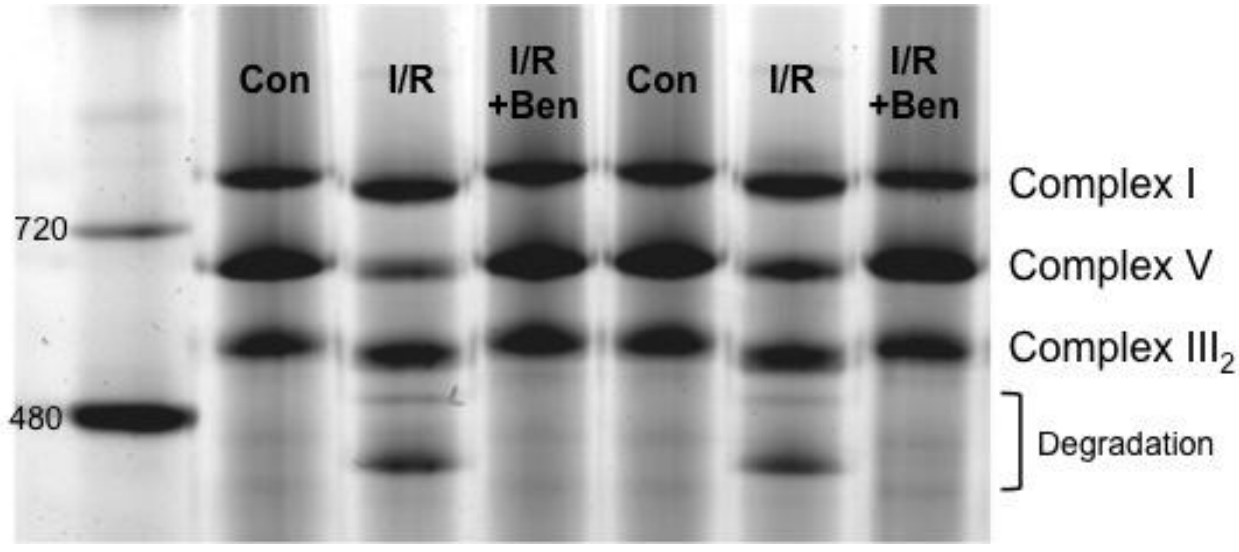

1100
kDa
Con #1
I/R #1
I/R +Ben #1
Con #2
I/R #2
I/R +Ben #2
Con #3
I/R #3
I/R +Ben #3
Gel #1
Con #4
I/R #4
I/R +Ben #4
Con #5
I/R #5
I/R +Ben #5
Con #6
I/R #6
I/R +Ben #6
Gel #2
Control (n=6)
Ischemia-Reperfusion (n=6)
Ischemia-Reperfusion + Bendavia (n=6)
Supercomplex Band Density (AU)
4000
3000
2000
1000
0
*

Figure 12 (cont'd)

B.



C.

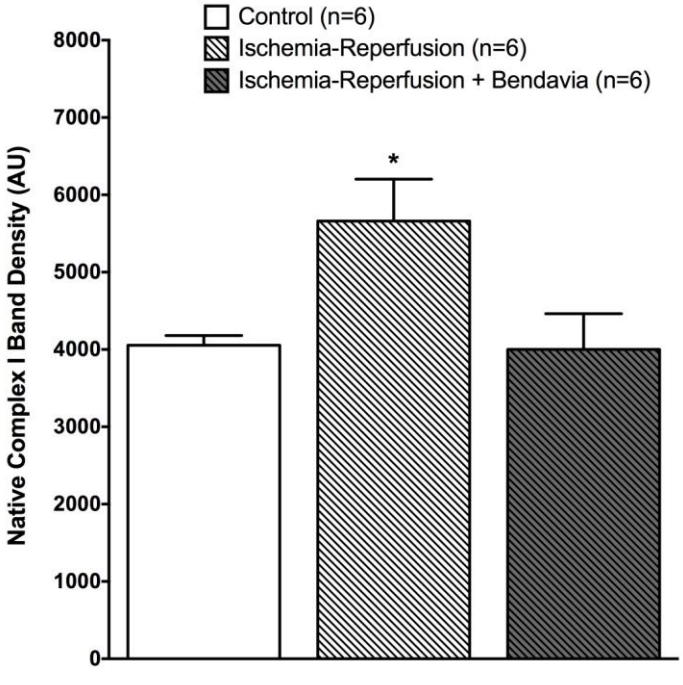
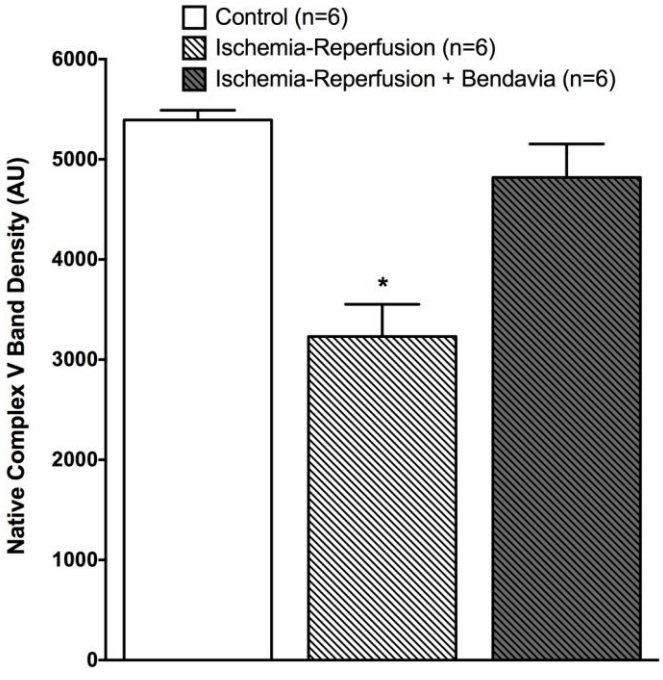


Figure 12 (cont'd)

D.



E.

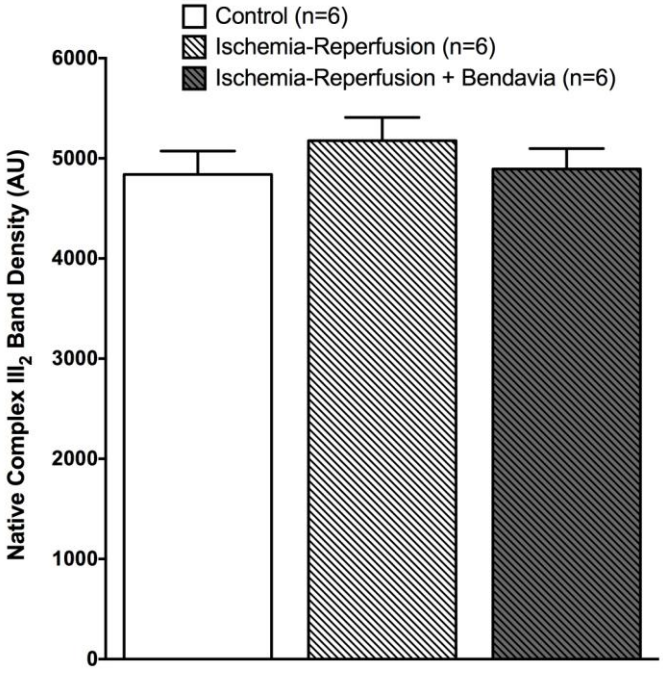


Figure 12 (cont'd)

F.

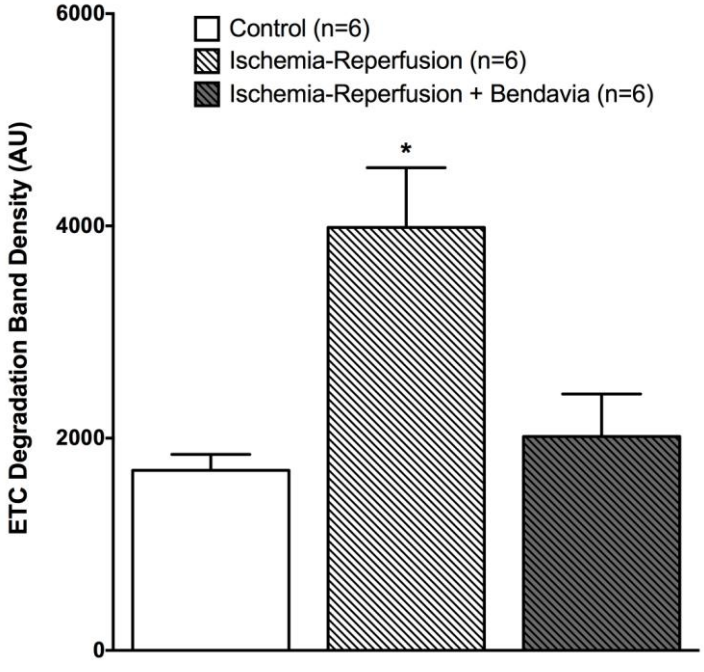
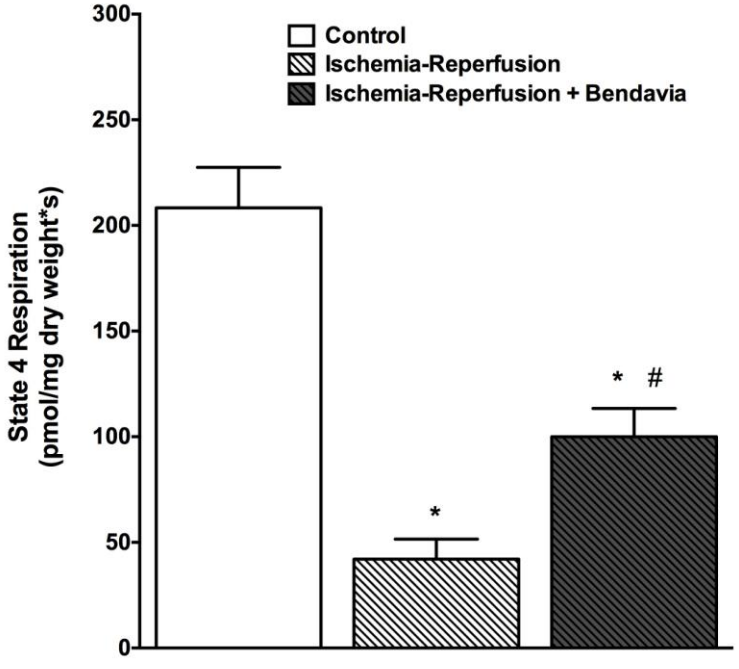


Figure 12. Blue-native gels for hearts in the study. **A.** Mitochondrial respiratory supercomplexes following ischemia-reperfusion. **B**. Representative BN-PAGE gel for various native electron transport system complexes. Quantification of native complex I (**C**), V (**D**), III_2 (**E**), and degradation bands (**F**) from hearts in the study. *, $P<0.05$.

Figure 13

A.


Control
Ischemia-Reperfusion
Ischemia-Reperfusion + Bendavia
State 4 Respiration (pmol/mg dry weight*s)
300
250
200
150
100
50
0
*
* #

B.

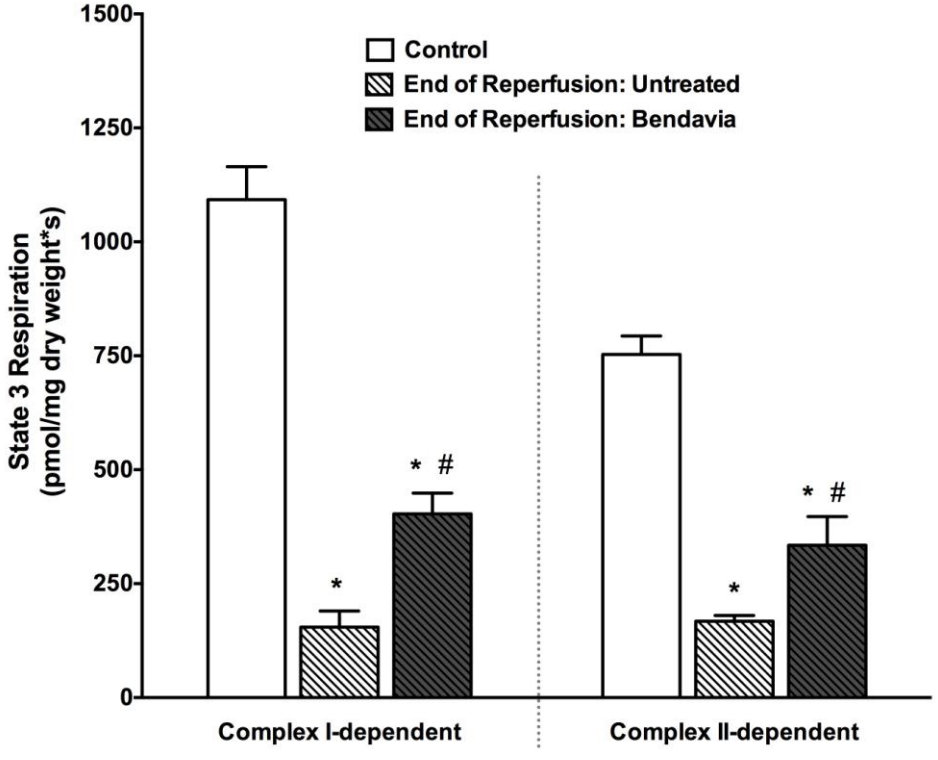
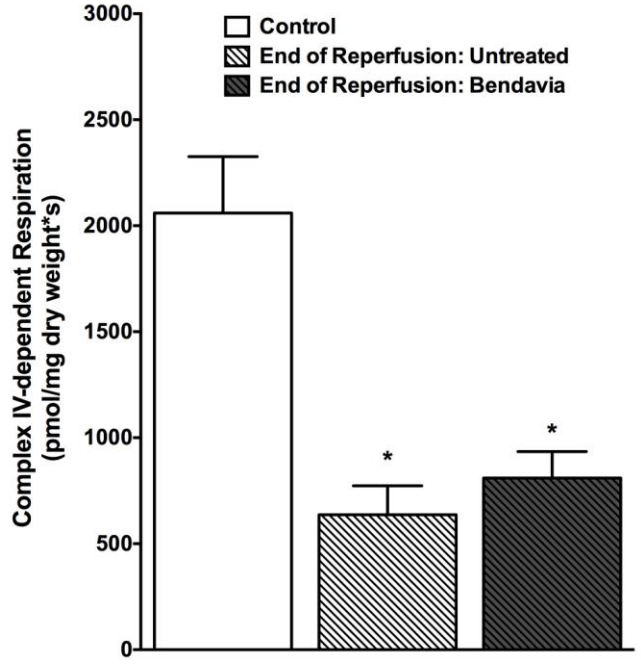

Control
End of Reperfusion: Untreated
End of Reperfusion: Bendavia
State 3 Respiration (pmol/mg dry weight*s)
1500
1250
1000
750
500
250
0
* #
*
* #
*
Complex I-dependent
Complex II-dependent

Figure 13 (cont'd)

C.



D.

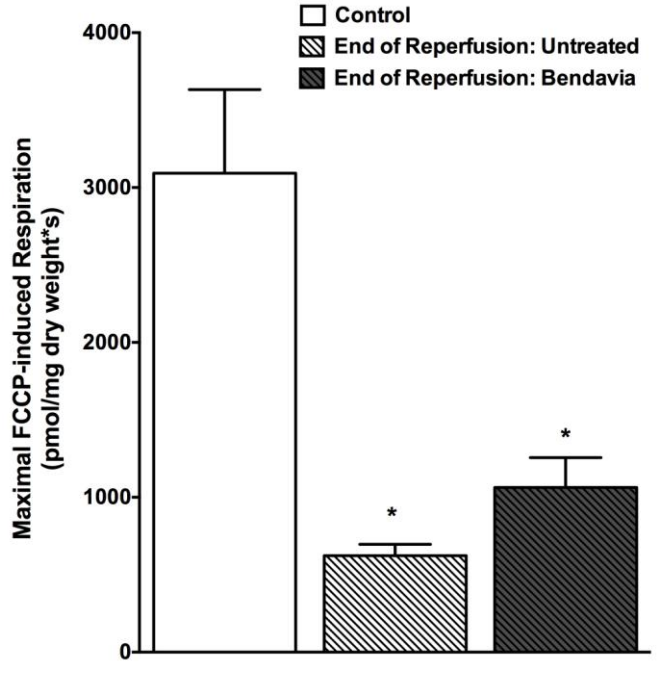


Figure 13. Site-specific changes in respiration following ischemia-reperfusion in permeabilized ventricular fibers. We employed a substrate-inhibitor titration protocol to assess mitochondrial

complex-dependent respiration. **A.** Rates of State 4 (uncoupled) respiration in hearts from the study (glutamate/malate substrate). **B.** State 3 respiration using either glutamate/malate or rotenone/succinate from hearts in the study. **C.** Respiratory rates for fibers using complex IV-dependent substrates. **D.** Maximal FCCP-induced respiration for ventricular fibers in the study. *, $P<0.05$ versus control; #, $P<0.05$ versus ischemia-reperfusion untreated.

Chapter 5: Integrated discussion

The work presented in this dissertation advances our understanding of the molecular basis of impaired cardiac function in two conditions: Diabetes and ischemia/reperfusion injury. We link decrements in mitochondrial bioenergetics of diabetic and ischemic hearts, to molecular and structural changes of key components in the energy-transducing inner mitochondrial membrane. Specifically, our studies uncover some of the molecular mechanisms that contribute to increased susceptibility to IRI (diabetic hearts) and to cardiac inefficiency (diabetic and post-ischemic hearts).

Our data, presented in chapter 2, show that the increased ischemic injury in diabetic hearts was associated with an enhanced susceptibility to PTP opening which was favored by an oxidative burden. We confirmed this oxidative burden by measuring the myocardial redox couple of glutathione (GSH/GSSG) which was significantly decreased in diabetic hearts compared to non-diabetic ones. In order to establish a relationship between the increased oxidative shift in the cellular redox state and the enhanced propensity to PTP opening in diabetic hearts, we used a combination of free thiols-labeling and immuno-precipitation of the PTP-regulatory protein, ANT. We were able to show that diabetic hearts had significant amounts of ANT that underwent post-translational oxidative modification, supporting the link between the oxidative shift in the redox state and the enhanced sensitivity to PTP-opening. Furthermore, our in vitro investigations of PTP opening under different redox states confirmed the redox-regulation of PTP opening. Indeed, we were able to delay PTP opening in diabetic mitochondria by shifting the redox state to a more reduced one by either using a reducing agent (DTT, in vitro) or the ROS-reducing peptide, Bendavia (in vitro and in vivo). In addition, our results from whole heart studies further confirmed the above findings and allowed us to link the increased ischemic

injury in diabetic hearts to PTP-opening. We were able to reduce the myocardial injury, as measured by infarct size, by inhibiting PTP opening either directly or indirectly. Indeed, we inhibited PTP opening at reperfusion using three different molecules: NIM811 (inhibit cyclophilin D, component of PTP), minocycline (inhibits MCU and reduces Ca overload that contributes to PTP opening) and Bendavia (reduces ROS generation that also contributes to PTP opening). All three approaches brought the infarct size in diabetic hearts to levels similar to those of non-diabetic hearts, suggesting that PTP opening is a major pathological factor in the enhanced susceptibility of diabetic hearts to IRI. Thus, our results presented in chapter 2 provide a mechanism for the increased susceptibility of diabetic hearts to IRI and most importantly, they show that this susceptibility can be overcome by targeting the mitochondria and inhibiting either one of the three major pathological factors (Ca overload, ROS or PTP opening).

Multiple studies have reported that diabetic cardiac mitochondria have a reduced capacity to accumulate Ca [58, 59, 69] and our data, presented in chapter 2, is in agreement with these reports. The fact that diabetic mitochondria have an enhanced sensitivity to PTP opening coupled with a reduced ability to accumulate Ca led Oliveira and colleagues [69] to conclude that this reduced capacity of mitochondrial Ca accumulation is the result of a defense mechanism rather than the result of defects in Ca uptake machinery [69]. However, Tanaka and colleagues [58] characterized the kinetics of mitochondrial Ca uptake and showed that diabetic cardiac mitochondria accumulated Ca at a slower rate and to a lower maximal extent suggesting that the machinery responsible for Ca uptake is altered by diabetes. Taking these results into consideration we hypothesized that the decreased ability of diabetic mitochondria to accumulate Ca is the result of molecular alterations of Ca uptake machinery (MCU complex). In addition, given the important role of Ca in the regulation of cardiac energy supply-demand matching, we

further hypothesized that these molecular alterations at the level of the MCU complex, if they exist, might explain the known mismatch between energy supply and demand in diabetic hearts.

We first wanted to establish the energy supply-demand mismatching in diabetic hearts by evaluating myocardial oxygen consumption (index of ATP generation) under different workload conditions. Our results in the Langendorff system presented in chapter 3 show that myocardial oxygen consumption was significantly decreased in diabetic hearts at baseline. Moreover, when we increased cardiac workload by pacing the hearts, the diabetic hearts were slower at adjusting their oxygen consumption to the changing demand and were unable to further increase their oxygen consumption to match higher workloads. These data might be explained by a slower mitochondrial Ca uptake that does not allow diabetic hearts to rapidly signal their energy needs to mitochondria, resulting in a limited capacity of energy matching. These data are in agreement with those reported for mice lacking MCU that exhibit a marked impairment in their ability to perform strenuous work [56].

In order to determine if there was an association between this energy supply-demand mismatching and mitochondrial Ca handling machinery, we next looked at MCU complex expression. Our data, presented in chapter 3, show a significant decrease in the expression levels of MCU and its regulatory protein MCUR1. The decrease in MCU could explain the inability of diabetic mitochondria to accumulate Ca to levels similar to those of healthy mitochondria. This is likely true, especially in the light of new evidence on the importance of MCU in mitochondrial Ca uptake that was provided by the use of transgenic mice lacking MCU [56]. Indeed, data reported in the study by Pan and colleagues [56] show that mice lacking MCU are unable to rapidly uptake Ca, compared to animals with intact MCU. Furthermore, our results also show a significant decrease in MCUR1, a protein that was recently reported to be essential for

mitochondrial Ca uptake and which ablation leads to the disruption of oxidative phosphorylation resulting in lower ATP levels [55]. These alterations of MCU and MCUR1 could explain, at least in part, the decline in mitochondrial Ca uptake by diabetic cardiac mitochondria and the directly related energy-supply demand mismatching seen in diabetic hearts.

We further characterized the bioenergetics of diabetic hearts by assessing the mitochondrial function in permeabilized cardiac fiber bundles using high resolution respirometry. Our results (chapter 3) show that complex I- and complex II-supported respirations were decreased in diabetic mitochondria under non-phosphorylating (complex I) and phosphorylating conditions (complexes I and II). In addition, the decrease in complex I-supported respiration was concomitant with a decrease in complex I subunit NDUFB8 as tested by western blot. We also found a significant decrease in the expression level of the ATP-synthase (complex V) in diabetic hearts. The latter can be another contributing factor to the energy supply-demand mismatching of diabetic hearts.

Taken together, these molecular changes, coupled with the functional defects that we showed in whole heart and permeabilized cardiac fiber bundles studies, provide a plausible mechanistic explanation for the decline in mitochondrial Ca uptake as well as the inefficient energy supply-demand matching in diabetic hearts.

Recent experimental evidence supports the idea that mitochondrial respiratory complexes (MRCs) are assembled into supramolecular organizations called supercomplexes or respirasomes. These assemblies are proposed to be the ultimate structural model for an efficient electron transfer between the redox components of the MRCs leading to an optimal energy generation and limiting electrons leak and ROS generation (reviewed in [45]). Since the focus of the work presented in this dissertation was to understand the molecular defects leading to supply-

demand mismatching in cardiac bioenergetics, whether it was caused by diabetes or IRI, we wanted to further characterize these defects in cardiac bioenergetics by looking at another level of organization of the MRCs: Supercomlexes. By focusing on the post-ischemic heart, we implemented and optimized the BN-PAGE approach to look at the expression of supercomplexes in mitochondria isolated from healthy and ischemic hearts. We performed these molecular analyses along with functional assessments of the cardiac mitochondria in permeabilized fiber bundles. We employed a substrate-inhibitor titration protocol to establish how each component of the ETC was influenced by ischemia-reperfusion. Our results, presented in chapter 4, show that ischemia-reperfusion led to a significant decrease in the overall mitochondrial function (complexes I-, II- and IV-supported respirations were decreased) with a pronounced reduction in supercomplex content. Furthermore, the decrease in supercomplex content in diabetic hearts was concomitant with three noteworthy changes. The latter were observed when mitochondrial membranes were solubilized using a stronger detergent (DDM) that breaks protein-protein interactions and separate the MRCs into free complexes. Those three major changes were: 1) increase levels of the free form of complex I, 2) apparition of small molecular weight bands that might be degradation products and 3) a significant decrease in complex V (ATP synthase) content. It has been shown that complex I is unstable [186] and can undergo degradation [188] when it is not integrated into the supercomplex assemblies. This could explain the apparition of degradation bands but does not explain the increased levels of complex I. A more quantitative approach such as mass spectrometry should be applied in order to understand this discrepancy.

Taken together these data suggest that following IRI, the structure (native gel data) and activity (respirometry data) of complex I are altered leading to an overall decrease in its oxidative capacity. These changes in complex I might be due to its non-optimal integration into

supercomplexes. Further analysis of the composition of supercomplexes is warranted in order to confirm this relationship that we are proposing.

We have shown in chapter 2 that targeting the mitochondria at reperfusion can confer cardioprotection. This led us to further investigate the effect of Bendavia on mitochondrial bioenergetics of diabetic and ischemic hearts. In diabetic hearts (chapter 3), this mitochondria-targeting peptide was able to improve myocardial oxygen consumption (at baseline and at lower workloads), to preserve mitochondrial Ca handling proteins (MCU and MCUR1) as well as complex I and ATP-synthase. These molecular effects were in agreement with the functional (respirometry studies) effects of Bendavia. In ischemic hearts (chapter 4), administration of Bendavia at reperfusion preserved supercomplexes and ATP-synthase contents. Furthermore, the hearts treated with Bendavia showed a higher recovery of mitochondrial function (respirometry studies).

This work identifies new mitochondrial targets of Bendavia: MCU, MCUR1 and different MRCs as well as their supramolecular organizations, respirasomes. Whether Bendavia acts directly or indirectly on these targets remains to be determined. However, the overall improved mitochondrial function and the preservation of multiple proteins of the inner mitochondrial membrane could, potentially, be explained by the action of Bendavia on a common denominator to all this targets. A potential common denominator that is important for the structure and function of multiple respiratory complexes as well as the respirasomes is cardiolipin. This idea of Bendavia targeting cardiolipin is supported by the newly proposed interaction between cardiolipin and Bendavia [44]. Further studies are warranted to unravel the interaction between Bendavia and cardiolipin in diabetic and ischemic hearts.

Taken together, the results presented herein provide new insight into the molecular and functional alterations that occur along the mitochondrial inner membrane in diabetic and post-ischemic hearts. These data provide a basis for novel therapies targeting the inner mitochondrial membrane as viable pharmacological approaches to improving bioenergetics in diseased myocardium.

Limitations

One limitation to our studies (presented in chapters 2 and 3) is the use of STZ-induced diabetes model which mimics more closely Type I diabetes. However, this model presents multiple pathological characteristics in common with Type II diabetes in both humans and animal models. Hearts from STZ-induced diabetic rats display alterations in myocardial calcium handling, substrate utilization, mitochondrial energetics, and enhanced oxidative stress (recently reviewed in [19]). So for the pathological aspects investigated in this work STZ model of diabetes was adequate. Another limitation is that we did not directly link mitochondrial Ca uptake to the energy supply-demand mismatching in whole hearts. Although we have tried to do so using spermine to activate MCU and see whether we could improve myocardial oxygen consumption in diabetic hearts, we obtained inconsistent results and decided to not pursue that approach.

Future directions

In chapter 3, we showed an association between reduced levels of MCU, MCUR1 and energy-supply demand mismatching in diabetic hearts. Future experiments are necessary to establish a causative relationship between MCU/MCUR1 and the energy supply-demand mismatching. For MCU, this can be easily accomplished using MCU-knockout mice and looking at their ability to match energy supply to demand with increasing cardiac workload. The activity

of Ca-sensitive dehydrogenases can also be examined and related to oxidative phosphorylation and ATP synthesis.

In chapter 4, we showed that supercomplexes content was reduced in post-ischemic hearts and was brought back to control levels when the hearts were treated with Bendavia at reperfusion. Future studies should determine whether these molecular changes translate into functional changes as well. Since the supercomplexes are in their native states, we can measure their enzymatic activities as well as their respiratory capacity.

Future studies are also needed to analyze the supercomplexes bands in order to determine: 1) the nature and stoichiometry of the respiratory complexes (I, II, III_2 or IV_{1-4}) and 2) the content and nature of cardiolipin in these supercomplexes. The results of these studies will allow us to determine whether the decrease in the content of supercomplexes is due to the dissociation of specific respiratory complexes (as we think is happening for complex I since the free form is increased) or it is due to a non-optimal assembly because of loss of cardiolipin.

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Appendix C: Animal care and use protocol approval

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

August 13, 2010

David Brown, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Brown:

Your Animal Use Protocol entitled, "Susceptibility to Cardiac Dysfunction in the Diabetic Heart," (AUP #Q292) was reviewed by this institution's Animal Care and Use Committee on 8/13/10. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

Appendix D: Animal care and use protocol amendment

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 13, 2010

David Brown, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Brown:

The Amendment to your Animal Use Protocol entitled, "Susceptibility to Cardiac Dysfunction in the Diabetic Heart", (AUP #Q292) was reviewed by this institution's Animal Care and Use Committee on 12/13/10. The following action was taken by the Committee:

"Approved as amended"

****Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

Appendix E: Animal care and use protocol amendment

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

May 31, 2011

David Brown, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Brown:

The Amendment to your Animal Use Protocol entitled, "Susceptibility to Cardiac Dysfunction in the Diabetic Heart", (AUP #Q292) was reviewed by this institution's Animal Care and Use Committee on 5/31/11. The following action was taken by the Committee:

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Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

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Appendix F: Animal care and use protocol approval

East Carolina University

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

June 22, 2012

David Brown, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Brown:

Your Animal Use Protocol entitled, "Cardiac Mitochondrial Calcium Handling and Energy Supply-Demand Mismatch in the Diabetic Heart" (AUP #Q309) was reviewed by this institution's Animal Care and Use Committee on 6/22/12. The following action was taken by the Committee:

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Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

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