The Physiological and Pathological Role of Mitochondrial Calcium in the Diabetic Heart

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DEPARTMENT OF KINESIOLOGY

Diabetic patients are more susceptible to ischemia/reperfusion injury and cardiac dysfunction likely due alterations in mitochondrial calcium handling. The purpose of this work was to determine if redox-dependent changes in permeability transition pore opening and mitochondrial calcium transients contribute to augmented injury and dysfunction in diabetic hearts. Langendorff-perfused streptozotocin-induced diabetic hearts were more susceptible to ischemia/reperfusion injury, with infarct sizes of $60\pm4\%$ of the area-at-risk (vs. $46\pm2\%$ in non-diabetics; $P<0.05$). Administration of 5uM NIM811 (non-immunosuppressive derivative of cyclosporine A), 1nM Bendavia (mitochondria-targeted antioxidant) or 1 uM Minocycline (blocker of mitochondrial Ca influx) at the onset of reperfusion reduced diabetic infarct sizes ($P<0.05$). Mitochondria isolated from the left ventricles of diabetic rats displayed greater sensitivity to Ca-induced permeability transition pore opening ($P<0.05$). Mitochondrial Ca uptake was slower in diabetic when compared to non-diabetic mitochondria ($P<0.05$), and Na/Ca exchange activity was faster in diabetic when compared to non-diabetic, despite no differences in

respiratory control ratio and mitochondrial membrane potential between groups. Treatment of diabetic mitochondria with 2mM of the reducing agent dithiothreitol significantly decreased the sensitivity to PTP opening and normalized mitochondrial calcium uniporter activity to non-diabetic levels. These findings suggest that the augmented susceptibility to injury and enhanced cardiac dysfunction in the diabetic heart is mediated by redox-dependent shifts in mitochondrial calcium handling, and that three novel mitochondria-targeted compounds administered at reperfusion may be suitable adjuvant reperfusion therapies to attenuate injury in diabetic patients.

The Physiological and Pathological Role of Mitochondrial Calcium in the Diabetic Heart

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$\pm$ dP/dt	Maximal rate of contraction and relaxation
ADP	Adenosine diphosphate
ANT	Adenine nucleotide transferase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bpm	Beats per minute
BSA	Bovine serum albumin
Ca	Calcium
CsA	Cyclosporine A
CyP-D	Cyclophilin D
DTT	Dithiothreitol
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
Em	Emission
Ex	Excitation
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.p.	Intraperitoneal
IR	Ischemia-reperfusion
IS	Isolation solution
KX	Ketamine/xylazine

LVDP	Left ventricular developed pressure
MCU	Mitochondrial calcium uniporter
mNCX	Mitochondrial sodium/calcium exchanger
MRI	Magnetic resonance imaging
MVO_2	Myocardial oxygen consumption
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
Pi	Inorganic phosphate
PiC	Mitochondrial phosphate carrier
PTP	Permeability transition pore
R	Gas constant
RCR	Respiratory control ratio
ROS	Reactive oxygen species
RyR2	Ryanodine receptor, cardiac muscle isoform
SERCA	Sarco/endoplasmic reticulum Ca-ATPase
SR	Sarcoplasmic reticulum
STZ	Streptozotocin
T	Temperature
TPP^+	Tetraphenylphosphonium ion
TTC	Triphenyltetrazolium chloride
v	Mitochondrial matrix volume
V	Buffer volume
VF	Ventricular fibrillation
VT	Ventricular tachycardia
$\Delta\Psi_m$	Mitochondrial membrane potential

Chapter 1

The Physiological and Pathological Role of Mitochondrial Calcium

in the Diabetic Heart

Introduction

Worldwide, an estimated 220 million people suffer from diabetes, and this number is expected to more than double within the next two decades (83). In the United States, eight percent of the population is diabetic, and nearly 80 million individuals are considered to be pre-diabetic (84). The rising population of diabetics (particularly in areas such as eastern North Carolina) has resulted in an enormous economic impact, with the total costs associated with diabetes nearing a staggering $200 billion annually (84). The fiscal crisis associated with diabetes is largely due to the host of health complications experienced by these individuals. In particular, diabetic patients are at an increased risk for developing heart disease (31) and are four times more likely to die following a myocardial infarction than non-diabetics (153). Moreover, hyperglycemia independently increases the risk for mortality (220).

In addition to the increased risk for mortality following myocardial infarction, diabetic patients experience cardiac dysfunction, independent of coronary artery disease or hypertension. As early as the 1970s, investigators observed that some diabetic patients suffering from heart failure had no sign of coronary artery disease or hypertension (98, 193). This generalized condition was termed *diabetic cardiomyopathy* by Rubler and colleagues (193), and presently includes a number of characteristic manifestations, including: systolic dysfunction and diastolic dysfunction, left ventricular

hypertrophy, impaired contractile reserve, as well as interstitial fibrosis [reviewed in (30)].

The mechanisms responsible for the increased incidence of cardiac dysfunction and enhanced propensity for death following myocardial infarction in diabetics are not clearly understood. However, insight provided by this work will shed light on these mechanisms and may promote the development of novel therapeutic strategies seeking to improve cardiac function and decrease the likelihood of heart failure in diabetic patients. Among the candidate mechanisms, a growing body of literature indicates that alterations in mitochondrial function may be responsible for the increased cardiac dysfunction and propensity for death observed in diabetes [reviewed in (30, 41)]. Specifically, diabetic heart mitochondria display enhanced susceptibility to opening of the mitochondrial permeability transition pore (PTP) in both humans (8) and animal models (23, 174, 232), alterations in substrate utilization (43, 109), decreased metabolic enzyme activity (80), depressed respiration (7, 80, 131), altered calcium handling (80, 181, 215) and increased reactive (ROS) production (32, 45, 128, 234) coupled with a decreased capacity to buffer ROS (5, 148, 237). Chapters 2 and 3 of this work will provide insight into the mechanisms underlying the increased susceptibility to death following myocardial infarction and the impaired cardiac dysfunction observed in diabetic hearts, respectively.

Models of Diabetes

To date, there have been few studies directly investigating the effects of diabetes on mitochondrial function in the human heart (7, 8), likely due to the difficulty in obtaining adequate human cardiac tissue samples. Recently, Anderson et al. were the

first to demonstrate attenuated mitochondrial respiratory capacity in the presence of increased H_2O_2 production in permeabilized fibers from human atrial tissue (7). The majority of mechanistic studies have involved the use of animals such as db/db (leptin receptor mutation) or ob/ob mice (leptin deficient), zucker diabetic fatty rats (leptin receptor mutation), or streptozotocin (STZ)-induced diabetic rats. Most studies have been conducted using the STZ-induced model of diabetes due to the comparability to humans and other animal models of diabetic cardiomyopathy as well as the ease of diabetes induction (42). Similar to both humans (7, 8, 193) and animal models of type 2 diabetes [reviewed in (41)], hearts from STZ-induced diabetic rats display alterations in mitochondrial calcium handling (80, 215), abnormal shifts in substrate utilization (109), as well as enhanced oxidative stress (202, 234), characterized by increases in reactive oxygen species (ROS) production and a decreased capacity to buffer ROS. Specifically, STZ-induced diabetic heart mitochondria exhibit decreased mitochondrial calcium influx in the presence of impaired left ventricular function (80, 215) and an enhanced susceptibility to PTP opening (174). Further, STZ-induced diabetic heart mitochondria display increases in fatty acid oxidation and depressed glucose oxidation coupled with decreased cardiac efficiency (109).

Ischemia-reperfusion injury and the consequences of mitochondrial Ca accumulation

In order to understand the mechanisms by which diabetic hearts are more susceptible to myocardial infarction, a brief overview of ischemia-reperfusion (IR) injury is provided. The first documented observation that coronary artery occlusion could lead to cessation of the heart beat was made in 1698 (48). Since that time, immense investigation has been conducted in order to determine the mechanisms by which

ischemia, and paradoxically reperfusion, lead to myocardial dysfunction and cellular injury. Following the onset of ischemia, myocardial contractility rapidly declines and this is accompanied by acidic pH, arrhythmogenesis, a decline in ATP production and cellular overload of Na and Ca (Figure 1.1). Irreversible cellular death begins between 15 and 20 minutes following the onset of ischemia, with the duration of ischemia correlating positively with clinical infarct size (area of dead tissue) (100). In addition, it is well established that infarct size is a predictor of both short- and long-term mortality (22, 86, 87, 105, 106).

While reperfusion remains the best treatment for ischemic injury, establishing reperfusion can lead to conditions that impair cardiac function. Following reperfusion of the tissue, the myocardium may experience stunning, fatal ventricular arrhythmias and/or post-ischemic cell death. Stunning is characterized by prolonged post-ischemic dysfunction of viable cells and may last for a few days (126, 127). In addition to stunning, fatal ventricular arrhythmias can ensue in early reperfusion due to mechanisms likely involving mitochondrial ROS production (35, 37).

The cellular death that occurs following IR is multi-factorial. Although many of the pathways are inter-related, the 'cause of death' for cardiac cells can include loss of membrane integrity, apoptosis, necrosis and autophagy [reviewed in (83)]. Our work presented herein will focus on the contribution of PTP-dependent IR injury, and the mechanisms that influence PTP opening. During cardiac ischemia, the loss of energetics leads to a significant rise in cytosolic Ca as ATP-dependent Ca pumps can no longer sequester cytosolic Ca, but the PTP remains closed due to acidic pH. At the onset of reperfusion, the now-polarized mitochondria provide a sink for the accumulated

intracellular Ca, while the surge of oxygen availability leads to a concomitant burst in mitochondrial ROS production. Thus at the onset of reperfusion when mitochondrial Ca content and ROS production are high, the conditions for PTP opening are ideal, resulting in mitochondrially-driven apoptosis and necrosis, with potentially irreversible cardiac damage (96). Of clinical interest, PTP opening has also been implicated in brain (50), renal (67), liver (123) and lung (59) IR injury as well as in diseases such as Alzheimer's (73) and cancer (82). Furthermore, the vast majority of cardiac patients experiencing IR arrive in the clinic after the onset of symptoms. Compounds that are effective when administered at reperfusion have enormous clinical potential. In Chapter 2, we provide evidence for at least two novel therapies that effectively reduce infarct size when given at the onset of reperfusion.

Mitochondrial Permeability Transition Pore in the Diabetic Heart

The PTP is a non-selective pore that forms within the inner membrane of mitochondria, allowing for the exchange of molecules less than 1.5 kDa (54, 103). The molecular components of the PTP are controversial, however, there appears to be an important role for the mitochondrial phosphate carrier (PiC) (134), and perhaps more importantly, the peptidly-prolyl cis-trans isomerase, cyclophilin-D (CyP-D) (216).   In addition, a regulatory role for the adenine nucleotide transferase (ANT) seems apparent (97), while knockout studies have ruled out a role for the voltage-gated anion channel (VDAC) (16). Halestrap and colleagues proposed a model for the PTP (Figure 1.2) where the PiC and ANT may form a heterodimer in the inner membrane of mitochondria. This process is proposed to be catalyzed by CyP-D and initiated during conditions of ischemia/reperfusion.   The opening of the PTP is favorable under

conditions where mitochondrial Ca is high, and the sensitivity to Ca-induced PTP opening is enhanced by alkaline pH, depletion of adenine nucleotides, increases in inorganic phosphate (P_i) and likely of most importance, oxidative stress [reviewed in (96)].

Diabetic heart mitochondria exhibit an enhanced susceptibility to opening of the PTP in both humans (8) and animal (23, 174, 232) models of diabetes. Therefore, it is logical that the increased susceptibility to PTP opening observed may be responsible for the increased proclivity for death following myocardial infarction in diabetes, however, this link has yet to be established. Oliveira and colleagues have shown that the probability of PTP opening in isolated diabetic heart mitochondria can be normalized to non-diabetic controls with direct inhibition of PTP opening utilizing cyclosporine A (CsA) (174), suggesting that blocking PTP opening may potentially attenuate IR injury in diabetic hearts. Therefore, it is plausible that inhibition of PTP opening may reduce the likelihood for PTP opening and provide protection against IR injury in diabetic hearts. Further, the mechanisms responsible for the increased susceptibility to PTP opening in diabetic heart mitochondria have not been revealed. Because oxidative stress significantly amplifies Ca-induced PTP opening, the oxidative shift in redox state characteristic of the diabetic heart may explain the increased proclivity for PTP opening. Chapter 2 will describe how a reductive shift in redox state can decrease the sensitivity to PTP opening in isolated diabetic heart mitochondria.

Pharmacological Inhibition of PTP Opening by Targeting Cyclophilin-D

Because PTP opening is a distinctive feature of IR injury, chemical inhibitors that prevent/delay PTP opening show promise in protecting against injury. Strategies that

target CyP-D have been frequently employed to inhibit PTP opening due to the notion that specific targeting of ANT and/or PiC would be deleterious to bioenergetic function. To date, a preponderance of studies have inhibited PTP opening by utilizing pharmacologic or genetic ablation approaches targeting CyP-D. A number of studies have indicated that inhibition of CyP-D with cyclosporine A (CsA) can decrease the probability of PTP opening and attenuate several indices of myocardial IR injury, including: infarction (10, 11, 101, 154, 228), left ventricular dysfunction (91, 102, 172), cardiomyocyte death (124, 165) and mitochondrial dysfunction (70, 173).

In a recent clinical trial (182), the use of CsA at the time of reperfusion reduced infarct size in humans by approximately 30% (when assessed by MRI), corroborating previous data from animal studies. Although promising, the use of CsA is confounded by a narrow therapeutic window (165), potentially harmful effects to the microvasculature (180, 214, 231), deleterious effects on long-term myocardial function (132), suppression of mitochondrial respiration (11), lack of protection against arrhythmia (4, 34), immunosuppression (15) and nephrotoxicity (163). Because of these potential adverse effects, the use of CsA should be particularly avoided in diseased populations that may be susceptible to these conditions. For example, diabetic patients experience depressed immune function (157) as well as high rates of kidney failure (2, 188). In fact, diabetes is the leading cause of renal failure (2). Due to the immunosuppressive and nephrotoxic effects of CsA, its use in the diabetic population would likely be precluded.

NIM811 is a non-immunosuppressive derivative of CsA developed by Novartis, and unlike CsA, NIM811 blocks PTP formation by selectively binding matrix CyP-D and

not the cytosolic immunosuppressant, cyclophilin A (222). NIM811 has been shown to decrease infarct size and directly block Ca-induced PTP opening in intact rabbit hearts and isolated rabbit cardiac mitochondria, respectively (12), by specifically binding CyP-D. NIM811 may prove to be a useful therapeutic tool for the treatment of myocardial IR injury, and Chapter 2 describes our use of NIM 811 to attenuate injury in the diabetic heart.

"Upstream" Inhibitors of the Mitochondrial Permeability Transition Pore

Given the relationship between mitochondrial Ca overload and PTP opening, blocking mitochondrial Ca influx can also decrease the open probability of PTP and decrease myocardial IR injury. Several blockers of the MCU, namely ruthenium red (RR) and ruthenium 360 (Ru360) (89), have been shown to protect the heart from IR injury (46, 85). However, the use of RR and Ru360 are confounded by non-specific Ca-blocking effects (120, 192) that may impair systolic and potentially diastolic function. These compounds also show membrane impermeability (21, 189), which would decrease the likelihood of intracellular entry of the drug into target tissues. The tetracycline antibiotic, minocycline, is just as effective in blocking MCU as Ru360 (Figure 1.3). Minocycline has been shown to diminish cardiac IR injury (190, 195) and has high permeability in cardiac tissue (190), making it an attractive candidate for protecting cardiac tissue during reperfusion by reducing mitochondrial calcium overload.

Due to the powerful effects of oxidative stress on enhancing PTP formation, it is no surprise that scavengers of free radicals (24, 146, 185, 197) have been shown to decrease the likelihood of PTP opening and provide protection. An investigation by Rajesh et al. revealed a significant decrease in myocardial infarct size in rats that

received the antioxidant MCI-186 30 minutes prior to a 30-minute ischemic insult (185). Other studies have employed the use of Mn superoxide dismutase (MnSOD) mimetics such as M40403 (146) and SC-52608 (24), each of which decreased myocardial IR *in vitro* and *in vivo*, respectively. Investigators have also utilized glutathione (GSH) (197) or the GSH precursor, N-acetyl cysteine (NAC), (68) and observed decreases in IR injury. While these data are promising, antioxidants have provided disappointing outcomes in clinical patients (3, 79, 140, 159, 240). Because the chief site of intracellular ROS production is within the mitochondria (213), antioxidants that target mitochondria would provide the greatest efficacy in reducing ROS during times such as IR when the ROS burden is high. A recent review by Murphy and Smith discusses the potential use of antioxidants conjugated to the lipophilic cation triphenylphosphonium (TPP$^+$) (161). The lipophilic and cationic properties of TPP$^+$ would allow the conjugated antioxidants to penetrate biological membranes and accumulate in mitochondria, but one limitation to their use is that uptake is based on mitochondrial membrane potential, which may be collapsed in early reperfusion (when the drug uptake is needed the most).

Recently, the antioxidant peptide Bendavia (SS31 in the literature) has been shown to attenuate myocardial IR injury (49). Bendavia has been shown to diminish ROS in several tissues (205), and has the unique property of targeting to mitochondria whether they are polarized or not (H Szeto, unpublished observations). Thus, Bendavia would provide efficient scavenging at the source of greatest ROS production (213). Our group has shown that administration of Bendavia at reperfusion (across a wide concentration spectrum) provides protection against IR injury (Figure 1.4).

Preconditioning as a Method of Protection in Ischemia-Reperfusion Injury

A large number of agents can reduce injury when given before ischemia, a general phenomenon called cardiac preconditioning. Preconditioning tissue with several sub-lethal bouts of ischemia before a long ischemia can reduce myocardial IR injury [first described in 1986 by Murry et al. (162)]. These investigators found that four cycles of five minutes of ischemia and five minutes of reperfusion immediately prior to a 40-minute ischemic insult in dogs could provide cardioprotection (measured by a 25% decrease in infarct size) when compared to non-preconditioned hearts. The cardiprotection provided by preconditioning was not accompanied by differences in coronary flow, suggesting that other cellular mechanisms were responsible for the cardioprotective effect. There are two windows of protection afforded by preconditioning [reviewed in (113, 160, 207, 238)]. The first window of protection appears to be within one hour following the initiation of the first preconditioning ischemic episode, while the second window of protection appears to be approximately 24 hours following preconditioning. While the second window of protection appears to be due to alterations in gene expression and protein synthesis, the first window of protection is too short for such adaptations to take place. Therefore, it is widely accepted that the cardioprotection afforded by first-window preconditioning is due to changes in cellular signaling pathways (see previous reviews on preconditioning), some of which may involve down regulation of PTP (111).

Complimenting studies that demonstrate ischemic preconditioning can protect against IR injury, several studies have shown that a variety of drugs when given before an ischemic insult can provide protection (26, 27). Among the large number of pharmacological preconditioning agents, anesthetic-induced preconditioning (with

inhalants) is observed in a variety of tissues (20, 61, 116, 119, 183, 225) and in species ranging from laboratory mice (151), rats (119, 141), guinea pigs (187), rabbits (92), dogs (152, 227) and human patients undergoing surgery (20, 116). While the use of anesthesia-induced preconditioning is an unlikely candidate for the treatment of myocardial infarction, it does have implications for patients undergoing transplantation surgery, where tissues such as liver, kidney, lung and heart become ischemic. Chapter 4 of the current work will discuss the cardioprotective effects of the anesthetic cocktail, ketamine-xylazine, in isolated hearts from guinea pigs.

Physiological Calcium Handling in the Heart

While intracellular overload of Ca can lead to both necrosis and apoptosis (94-96), it is well established that Ca is a key regulator of both myocardial contraction and the ATP producing metabolic processes that support myocardial contraction (89, 90). Both cytosolic (28, 76, 130) and mitochondrial (80, 181, 215) Ca handling have been revealed to be impaired in the diabetic heart, which could explain a portion of the cardiac dysfunction observed in the diabetic condition. In order to understand how dysfunctional Ca handling in the diabetic heart contributes to cardiac dysfunction, a brief review of myocardial Ca handling is provided (Figure 1.5).

Under physiological conditions, a small amount of extracellular Ca enters the cardiac myocyte down its electrochemical gradient through the L-type Ca channels in a process initiated by sarcolemmal depolarization. Entry of extracellular Ca into the cytosol triggers the release of comparatively large quantities of Ca by the Ca-sensitive ryanodine receptors (RyR2) located on the sarcoplasmic reticular (SR) membrane in a process known as Ca-induced Ca-release. Ca then binds to troponin C and allows for

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the interaction of actin and myosin, initiating systole. In order for diastole to take place, cytosolic Ca must return to baseline levels (~100nM). The major routes for Ca uptake are the ATP-dependent sarco/endoplasmic reticulum Ca-ATPase (SERCA), sarcolemmal NCX and Ca-ATPase and mitochondria.

The critical role of Ca in the myocardium does not terminate at the level of contraction and relaxation. Ca is a vital activator of several key sites of NADH production and metabolic control within the mitochondria. Not only does Ca enter and leave the cytosol on a beat-to-beat basis, but it has also been suggested to enter the mitochondria on a beat-to-beat basis (189). Under physiological conditions, Ca enters the mitochondria down its electrochemical gradient through the mitochondrial Ca uniporter (MCU) (18, 64, 89) and exits in a sodium-dependent manner through the mitochondrial Na-Ca exchanger (mNCX) (54-56, 176) (Figure 1.6). Nearly 40 years ago, Ca was shown to both directly and indirectly activate NADH producing enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (66, 99, 167). In addition to activation of NADH production, Ca can directly contribute to the activity of complex V of the mitochondrial electron transport system. Even in the presence of NADH, the activity of the F_oF_1 ATPase is inhibited in the absence of Ca (217). Providing support for the activation of the NADH producing enzymes and the ATPase, increasing mitochondrial Ca levels leads to an increase in ATP synthesis, while decreasing mitochondrial Ca decreases ATP synthesis (115). Therefore, Ca entry into mitochondria is critical to ATP production and support of myocardial contraction. Due to the regulatory role of Ca for both contraction and ATP production, Balaban proposed the concept of "parallel activation" in myocardial supply

and demand matching (17). Alterations in Ca handling could impair the ability of mitochondria to meet the myocardial energy demand with the appropriate supply of energy. This energy mismatch can result in myocardial mechanical failure (i.e. depressed left ventricular ejection fraction, decreased ventricular compliance, etc.), thus, in such a situation, an adequate supply of nutrients may not be unable to reach systemic tissues.

Due to the important role of mitochondrial Ca in ATP production (115), recent evidence has suggested that mitochondrial Ca handling may be altered failing hearts (138, 139). A study by Liu and O'Rourke revealed that myocytes from a guinea pig model of heart failure had decreased levels of NADH, and treatment with CGP-37157 (inhibitor of mNCX) significantly increased NADH. These data suggest that an increase in mitochondrial Ca can improve mitochondrial bioenergetics in diseased hearts. This study supports previous work (115), suggesting that maintenance of mitochondrial Ca is integral to ATP production and that a decrease in mitochondrial Ca can result in heart failure.

Calcium Handling in the Diabetic Heart

Interestingly, many characteristics of diabetic hearts overlap with those of heart failure, including both systolic (75, 193) and diastolic dysfunction (33, 198, 200) as well as metabolic inflexibility (38, 147, 226). Diabetic hearts exhibit abnormal cytosolic Ca homeostasis, characterized by increased diastolic and/or decreased systolic Ca levels [reviewed in (135)]. A study by Allo et al. demonstrated that the activity of both the sarcolemmal Na-Ca exchanger and sarcolemmal Ca-ATPase were decreased in diabetic hearts (6). In addition, a study by Ganguly and colleagues provided evidence

of decreased SERCA activity coupled with impaired diastolic dysfunction (84). Complimentary to this study, overexpression of SERCA2a (subunit of the SERCA) improves impairments in contractility in diabetic mice (219), suggesting that dysfunctional cytosolic Ca handling may contribute to the cardiac dysfunction observed in diabetes, and cytosolic Ca levels have been shown to influence mitochondrial Ca (189). Therefore, aberrant cytosolic Ca handling observed in diabetic hearts may translate to changes in mitochondrial Ca. Moreover, the overlap in the characteristics of failing non-diabetic hearts with those of diabetic hearts (described above) may also suggest that mitochondrial Ca could be altered in diabetes. The recent observation by Liu and O'Rourke that mitochondrial Ca handling is altered in heart failure may translate to the diabetic heart. Previous studies have suggested that mitochondrial Ca influx is slower in isolated mitochondria from diabetic hearts (80, 181, 215), and Chapter 3 of this work will provide compelling direct evidence that mitochondrial Ca influx through MCU and Ca efflux via mNCX are altered in isolated diabetic heart mitochondria, in the absence of differences in $\Delta\Psi_m$.

Tanaka and colleagues demonstrated reduced Ca influx into diabetic mitochondria. However, the reduced mitochondrial Ca uptake observed in diabetic hearts was coupled with a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). Because $\Delta\Psi_m$ is the primary driving force for mitochondrial Ca influx, a decrease in $\Delta\Psi_m$ will decrease Ca influx into mitochondria. Therefore, it is difficult to determine whether the difference in mitochondrial Ca influx observed in this study was solely due to decreased $\Delta\Psi_m$ differences or if other factors were involved, such as altered expression of MCU or mNCX. Moreover, the $\Delta\Psi_m$ measurements were determined using the

lipophilic cation TPMP$^+$ in myocytes. When used in intact cells, the driving force for TPMP is a combination of mitochondrial and sarcolemmal membrane potentials. Given that diabetic hearts are well-known to have lower repolarizing K$^+$ currents (133), diminished TPMP uptake (as seen by Tanaka et al.) could be due to smaller sarcolemmal membrane potential and independent of $\Delta\Psi_m$. Flarsheim and coworkers also demonstrated a reduction in mitochondrial Ca influx in diabetic rat hearts (80). However, they did not measure $\Delta\Psi_m$ in this study. In addition, these observations were made using the Ca indicator, arsenazo III, which as been shown to enhance the production of ROS (155).

Based on the bioenergetic importance of mitochondrial Ca, decrements in the activity of these mitochondrial Ca pathways may be responsible for the impaired cardiac function and decreased cardiac efficiency observed in both human (178, 179) and animal models of diabetes (31, 32). Because supply-demand mismatch is a distinguishing feature of clinical heart failure (60, 62, 166), it is logical that the impaired mitochondrial Ca handling observed in diabetes may be responsible for the cardiac dysfunction observed. While there have been mechanistic studies to explain alterations in cytosolic Ca handling in diabetic hearts (219, 230), until this work, there has been no mechanistic insight into the alterations observed in mitochondrial Ca handling. Diabetic heart mitochondria display an oxidative shift in redox state characterized by increased ROS production (32, 45, 128, 234) and a decreased capacity to buffer ROS (5, 148, 237). In particular, diabetic hearts display a decrease in the activity of glutathione peroxidase and Cu/Zn superoxide dismutase (5) as well as impairments in catalase activity (237). Oxidative shifts in redox state can alter the activity of cytosolic Ca

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handling proteins (65, 218). For example, oxidative stress induces Ca leak from RyR2 (65, 218) in heart failure, and this leak can be reversed with the antioxidants vitamin C and E (65). No laboratories have investigated the effects of the oxidative shift in redox state characteristic of the diabetic heart on mitochondrial Ca handling. Chapter 3 of this work will provide cogent evidence that the oxidative shift in redox state characteristic of the diabetic heart directly impairs mitochondrial calcium handling, and this impairment can be normalized with a reducing agent.

Conclusions

The continuously growing diabetic population exhibits signs of heart failure, independent of coronary artery disease and hypertension. In addition, diabetics are more likely to die following myocardial infarction when compared to non-diabetics, and accordingly, ischemic heart disease is the leading cause of death among the rapidly growing diabetic population. Animal models of diabetes also exhibit increased proneness for cardiac injury, characterized by heightened propensity for electromechanical dysfunction and cell death. Cardiac mitochondrial dysfunction has been recently proposed as a culprit in the enhanced susceptibility of the diabetic heart to failure and ischemic injury. Specifically, diabetic heart mitochondria display 1) an increased propensity for PTP opening 2) impairments in mitochondrial Ca handling and 3) an oxidative shift in redox state. However, the impact of this shift in redox state on increased likelihood for PTP opening and impaired mitochondrial Ca handling has not been realized. In order to develop effective therapeutic strategies designed to mitigate cardiac dysfunction and decrease IR injury in the diabetic heart, the underlying cellular mechanisms must be elucidated.

Central Hypothesis

Chapter 2 will investigate the hypothesis that increased propensity for PTP opening characterized by diabetic hearts is responsible for the increased myocardial IR injury observed. We believe that the oxidative shift in redox state characteristic of the diabetic heart increases the likelihood for PTP opening and impairs mitochondrial Ca handling in diabetic hearts. It is further hypothesized that shifting the redox state towards a more reduced state will decrease the likelihood of PTP opening and improve mitochondria Ca handling in diabetic hearts.

The observations of this work support the hypotheses that 1) the increased susceptibility to PTP opening is responsible for the increased likelihood of myocardial IR injury in diabetes and 2) the oxidative shift in redox state characteristic of the diabetic heart is responsible for the increased propensity to PTP opening and the impaired mitochondrial Ca handling observed in the diabetic heart. Insights provided by this work may provide invaluable preventative measures or therapeutic treatments for diabetic patients that suffer myocardial infarction and/or heart failure.

Chapter 2

Increased Susceptibility to Mitochondrial Permeability Transition Pore

Opening in the Diabetic Heart

Abstract

Diabetic patients are more susceptible to ischemia/reperfusion (IR) injury, likely due to enhanced mitochondrial permeability transition pore (PTP) opening. The purpose of this study was to: 1) determine if three novel mitochondria-targeted compounds administered at the onset of reperfusion protect diabetic hearts from injury and 2) determine if redox-dependent changes in PTP opening contribute to augmented injury in diabetic hearts. Langendorff-perfused STZ-induced diabetic hearts were more susceptible to IR injury, with infarct sizes of $60\pm4\%$ of the area-at-risk (vs. $46\pm2\%$ in non-diabetics; $P<0.05$). Administration of 5uM NIM811 (non-immunosuppressive derivative of cyclosporine A), 1nM Bendavia (mitochondria-targeted antioxidant) or 1uM minocycline (blocker of mitochondrial Ca influx) at the onset of reperfusion reduced diabetic infarct sizes ($P<0.05$). Mitochondria isolated from the left ventricles of diabetic rats displayed greater sensitivity to PTP opening ($P<0.05$). Treatment of diabetic mitochondria with 2mM of the reducing agent dithiothreitol and 4 days of daily treatment with Bendavia significantly decreased the sensitivity to PTP opening. These findings suggest that the augmented susceptibility to injury in the diabetic heart is mediated by redox-dependent shifts in PTP opening, and that three novel mitochondria-targeted compounds administered at reperfusion may be suitable adjuvant reperfusion therapies to attenuate injury in diabetic patients.

Introduction

Presently, the United States is home to approximately 26 million diabetic individuals (84), and by the year 2025, it is estimated that the global population of diabetics will reach 300 million (125). Diabetes significantly increases the probability of death following myocardial infarction (153), with hyperglycemia independently increasing the risk for mortality (220). The underlying mechanisms responsible for augmented injury in diabetics are not clear. This paucity in the literature provides a substantial barrier for the development of effective therapeutic strategies seeking to diminish ischemic injury in diabetic patients. Elucidating the cellular mechanisms that contribute to the cardiac dysfunction and augmented ischemic injury observed in the diabetic condition may foster novel putative treatments for diabetic patients that experience myocardial infarction.

While the etiology for the increased risk of ischemic injury is undoubtedly multi-factorial, a growing body of evidence suggests that aberrant mitochondrial function plays a significant role in the pathogenesis of the increased proclivity for death observed in these patients [reviewed in (41)]. Specifically, diabetic heart mitochondria exhibit an increased propensity for mitochondrial permeability transition pore opening (PTP) (8, 23, 174, 232), coupled with an oxidative shift in redox state, characterized by increased reactive oxygen species (ROS) production (32, 45, 128, 234) and a decreased capacity to buffer ROS (5, 148, 237). Mitochondrial overload of Ca can lead to the emergence of the mitochondrial permeability transition pore (PTP), particularly under conditions of oxidative stress [reviewed in (93, 95, 96)]. PTP opening results in depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) and hydrolysis rather than generation of ATP,

followed by subsequent loss of cardiac electromechanical function and cell death. PTP induction is particularly favorable during ischemia/reperfusion (IR). During cardiac ischemia, the loss of energetics leads to a significant rise in cytosolic Ca as ATP-dependent Ca pumps can no longer reduce cytosolic Ca. At the onset of reperfusion, the now-polarized mitochondria provide a sink for the accumulated intracellular Ca, while the surge of oxygen availability leads to a concomitant burst in mitochondrial ROS production. Thus at the onset of reperfusion when mitochondrial Ca content and ROS production are high, the conditions for PTP opening are ideal.

Diabetic heart mitochondria demonstrate an enhanced susceptibility to opening of the PTP in both humans (8) and animal models of diabetes (23, 174, 232). It is certainly plausible that the increased susceptibility to PTP opening observed in the diabetic heart may be responsible for the increased proclivity for death following myocardial infarction. However, until this investigation, that link had yet to be established in diabetic hearts. Further, the mechanisms responsible for the increased susceptibility to PTP opening in diabetic heart mitochondria have not been revealed. Because oxidative stress significantly amplifies Ca-induced PTP opening, the oxidative shift in redox state characteristic of the diabetic heart (described above) may explain the increased proclivity for PTP opening. Here, we demonstrate that directly inhibiting PTP opening utilizing NIM811 (non-immunosupressive derivative of CsA) and indirectly inhibiting PTP opening with Bendavia (novel mitochondria-targeted antioxidant) and minocycline (blocker of mitochondrial Ca influx) reduces infarct size in isolated diabetic hearts and to the same extent as drug treated non-diabetic hearts. Further, we provide evidence that a reductive shift in the redox state of isolated diabetic heart mitochondria

decreases the sensitivity to Ca-induced PTP opening. The observations of this work suggest that the increased propensity for IR injury in diabetic hearts is due to enhanced susceptibility to PTP opening, and the shift in redox state characteristic of the diabetic heart increases the likelihood for PTP opening and impairs mitochondrial Ca handling.

Materials and Methods

All reagents used were of the highest grade commercially available (Sigma-Aldrich, United States). Calcium green 5N salt was purchased from Invitrogen (Carlsbad, CA, USA).

All animal studies were approved by the East Carolina University Institutional Animal Care and Use Committee and were in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*. 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 five 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 using a commercially available glucometer (One Touch Ultra 2, LifeScan, Milpitas, CA, USA

Beating hearts were removed from anesthetized (ketamine/xylaine; 85/15 mg/kg) rats via bilateral thoracotomy and perfused (perfusion pressure of 75 mmHg) in a retrograde fashion on a modified Langendorff apparatus using an established protocol

previously described by our group (204). 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 mmHg. Three electrodes were placed into the buffer-filled perfusion chamber for the measurement of volume-conducted ECG. A pre-established protocol of electrode placement was utilized to obtain a signal analogous to Lead II of a typical 12-lead ECG (34, 35). Coronary flow rates were monitored constantly with a flow probe (Transconic Systems, Ithaca, NY, USA) connected in series with the perfusion line. 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.

Following a 10 minute baseline period, ischemia/reperfusion was initiated similarly to that described previously by our group (204). Hearts were exposed to global no-flow ischemia by stopping perfusion for 20 minutes. 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 was allowed to ensue for 2 hours with or without the presence of 5 mM NIM811 [inhibitor of the PTP(11)], 1 nM Bendavia [mitochondrial-targeted antioxidant (210)] or 1 uM minocycline (blocker of mitochondrial calcium influx, Figure 1.4). Because treatment at the start of reperfusion is most clinically relevant, these drugs were introduced at the start of the 2-hour reperfusion protocol. At the end of reperfusion, the left ventricle was dissected, sliced into 5mm-thick slices, incubated in 1% triphenyltetrazolium chloride (TTC) for 10 minutes ($37°C$) and digitally photographed for subsequent infarct size analysis. TTC stained viable tissue bright red, while infarcted tissue appeared pale in color. Infarct size was expressed as the infarcted area as a percentage of the left ventricle (calculated using ImageJ software, NIH, Bethesda, MD, USA). Arrhythmias were scored as described previously by our group (34, 35, 204) and were in accordance with the Lambeth Conventions (57, 223). Arrhythmias were scored during the reperfusion period as follows: 0 = 0 − 49 ventricular premature beats; 1 = 50 − 499 ventricular premature beats; 2 = > 499 ventricular premature beats and/or 1 episode of spontaneously reverting ventricular tachycardia (VT) or ventricular fibrillation (VF) less than 60 sec in total duration; 3 = > 1 episode of VT or VF that is < 60 sec total duration; 4 = reverting VT or VF or both that is < 120 s in total duration; 5 = VT or VT or both that is > 120 sec in combined duration; 6 = non-reverting (fatal) VT or VF that began > 15 min after reperfusion; 7 = fatal VT/VF that began between 5 min and 15 min after reperfusion; 8 = fatal VT/VF that began less than 5 min after reperfusion.

Cardiac mitochondria were isolated from the left ventricle of hearts utilizing a protocol similar to Boehm et al. (25). 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. Rat hearts were excised from anesthetized rats (as described above) 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 minutes 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 (1mg/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 600g for 10 minutes, and the supernatant was then centrifuged at 8000g for 15 minutes. 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 mL IS buffer. Mitochondrial protein content was determined using a BCA protein assay.

To determine the quality of our mitochondrial preparations, respiratory control ratios (RCR) 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 K_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. RCRs were calculated by dividing the state 3 respiration by the state 2 respiration.

Mitochondria (0.75 mg) were suspended in mitochondria assay buffer (described above) and supplemented with the fluorescent probe 1 μM calcium green 5N salt (Ex = 506 nm, Em = 532) to track changes in extramitochondrial calcium. Fluorescence was measured using a spectrophotometer (Photon Technology International, Birmingham, NJ, USA). Calcium-induced permeability transition pore (PTP) opening experiments were performed under state 2 conditions. Mitochondria were energized with 5 mM glutamate/5 mM malate. Mitochondrial permeability transition pore opening was induced by subjecting mitochondria to 50 nmole $CaCl_2$ pulses at 3-minute intervals. PTP induction was denoted by the inability of mitochondria to take up calcium (sharp increase in extramitochondrial calcium fluorescence). Data were quantified as the amount of calcium needed to induce PTP opening (nmoles $CaCl_2$/mg mitochondria). For experiments where redox state was manipulated, energized mitochondria were treated with either 200 μM diamide or 2 mM dithiothreitol (DTT) for the 10 minutes prior to and throughout each experimental protocol. Previous work from our laboratory has indicated that this concentration of diamide can elicit oxidative stress by significantly lowering GSH/GSSG (35). Others have shown that this concentration of DTT can act as a reducing agent and decrease the likelihood of PTP opening under oxidative conditions (63).

A sub-set of rats (for PTP experiments) was treated daily with the mitochondria-targeted antioxidant, Bendavia (aka SS-31 in the literature). In this sub-set of STZ-induced diabetic rats and control rats, Bendavia[TM] (1.5 mg/kg in 0.9% saline) was injected (i.p.) daily for the 4 days prior to each mitochondrial isolation day.

Data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA or two-way ANOVA (as appropriate), with Newman-Keuls post-hoc analysis for comparison between groups. The level of significance was established at P < 0.05.

Results

Animal characteristics*.* Rat body weights, heart weights (corrected for body weight) and fasting glucoses are presented in Table 2.1. STZ treated rats had significantly lower body weights as well as lower heart weights when compared to non-STZ treated rats (P<0.05). In order to confirm diabetes in STZ treated rats, we measured fasting blood glucose. STZ treated rats displayed significantly higher fasting glucose levels when compared to non-STZ treated rats (495 ± 21.9 vs. 114 ± 1.43, respectively; P<0.05).

Infarct size. Infarct sizes and representative infarct pictures are presented in Figure 2.1. Langendorff-perfused hearts from STZ treated 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 5uM NIM811 (non-immunosuppressive derivative of cyclosporine A), 1nM Bendavia (mitochondria-targeted antioxidant) and 1 uM minocycline at the onset of reperfusion significantly reduced diabetic infarct sizes (P<0.05) and to the same extent as NIM811 and Bendavia treated non-diabetic animals (P>0.05).

Hemodynamics. Hemodynamics are presented in Tables 2.2. There were no significant differences in hemodynamic parameters before the ischemic period nor at the end of reperfusion (P>0.05). During the baseline and at the end of reperfusion, left

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ventricular developed pressure, maximal rates of contraction (+dP/dt) and relaxation (-dP/dt), and coronary flow rates were similar between all groups.

Arrhythmia scores. Arrhythmia scores are presented in Figure 2.2. There were no differences in the incidence arrhythmias as measured by infarct size.

Respiratory control ratio. Respiratory control ratios are presented in Figure 2.3. There were no differences in respiratory control ratios between mitochondria isolated from STZ and non-STZ treated rats (P>0.05), suggesting that the quality of mitochondrial preparation was not different between groups.

Calcium-induced PTP opening. Fluorescence data for Ca-induced PTP opening as well as representative traces are depicted in Figure 2.4. 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 or when animals received daily i.p. injections of 1.5 mg/kg Bendavia for the four days leading up to the experimental day. However, treatment with DTT and Bendavia did not normalize Ca-induced sensitivity to non-diabetic control levels. Treatment with 200 uM 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 (vs. respective controls), suggesting that the redox state of diabetic isolated mitochondria can not be oxidized further.

Discussion

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In this study, we hypothesized that the increased susceptibility to ischemia-reperfusion (IR) injury observed in diabetes is due to an increased probability for mitochondrial permeability transition pore opening (PTP). Moreover, we hypothesized that the increased propensity for PTP opening displayed by the diabetic heart was the result of an oxidative shift in the redox state. The major findings of this work are 1) direct inhibition of PTP opening with NIM811 and indirect inhibition of PTP with Bendavia and minocycline significantly reduce infarct size in isolated diabetic hearts to the same extent as drug treated non-diabetic control hearts and 2) four days of treatment with Bendavia and treatment of isolated diabetic heart mitochondria with dithiothreitol significantly reduce the sensitivity to Ca-induced PTP opening. To our knowledge, no previous study has described the cardioprotective effects of blocking PTP opening at the onset of reperfusion in diabetic animals. Moreover, we are the first to show that a reductive shift in the redox state of isolated diabetic heart mitochondria decreases the sensitivity to Ca-induced PTP opening.

Enhanced Sensitivity to PTP Explains Augmented IR Injury in Diabetic Hearts

Since first described nearly four decades ago (193), scores of investigators have attempted to uncover the mechanisms underlying diabetic cardiomyopathy [reviewed in (29, 30)]. Recently, an emerging body of evidence [reviewed in (41)] has pointed towards mitochondrial dysfunction as a potential culprit for the increased probability for death following myocardial infarction in these patients. In particular, diabetic heart mitochondria display an enhanced propensity for PTP opening (8, 23, 174, 232), and PTP opening is widely accepted to be a distinctive feature of IR injury. Because PTP opening is a hallmark of IR injury, inhibition of PTP opening provides substantial

protection against injury [reviewed in (96)]. However, until this work, no investigators had employed the use of PTP inhibition in diabetes to attenuate augmented myocardial IR injury.

Strategies that target cyclophilin D (CyP-D), a distinctive feature of the PTP, have been frequently employed to directly inhibit PTP opening (94-96). A number of studies have indicated that inhibition of CyP-D with cyclosporine A (CsA) can decrease the probability of PTP opening and attenuate several indices of myocardial IR injury in non-diabetic animals, including: infarction (10, 11, 101, 154, 228), left ventricular dysfunction (91, 102, 172), cardiomyocyte death (124, 165) and mitochondrial dysfunction (70, 173). In a recent clinical trial (182), the use of CsA at the time of reperfusion reduced infarct size in humans by approximately 30% (when assessed by MRI), corroborating previous data from animal studies. Although promising, the use of CsA is confounded by a narrow therapeutic window (165), potentially harmful effects to the microvasculature (180, 214, 231), deleterious effects on long-term myocardial function (132), suppression of mitochondrial respiration (11), lack of protection against arrhythmia (4, 34), nephrotoxicity (163), and immunosuppression (15). Due to the high rates of renal failure (2) and depressed immune function (157) in diabetic patients, we chose to employ the use of NIM811 to block PTP opening in isolated diabetic hearts. NIM811 is a non-immunosuppressive derivative of CsA developed by Novartis, and unlike CsA, NIM811 blocks PTP formation by selectively binding matrix CyP-D and not the cytosolic immunosuppressant, cyclophilin A (222). It is widely accepted that CyP-D initiates PTP opening, and NIM811 has been shown to decrease infarct size and directly block Ca-

induced PTP opening in intact non-diabetic rabbit hearts and isolated rabbit cardiac mitochondria, respectively (12), by specifically binding CyP-D.

A previous study demonstrated that the enhanced propensity for PTP opening in isolated diabetic heart mitochondria could be normalized to non-diabetic controls with CsA treatment (174). In order to determine whether inhibition of PTP opening could attenuate myocardial IR injury in diabetic whole hearts to the same extent as non-diabetic hearts, we utilized NIM811. Corroborating previous reports (9, 69, 110, 129, 144, 145, 199), we demonstrate that diabetic hearts are more susceptible to IR injury, as measured by a 30 percent increase in infarct size in our study. The significance of our work is that directly inhibiting PTP opening with NIM811 at the onset of reperfusion attenuates infarct size to the same extent as NIM811 treated non-diabetic hearts, suggesting that the increased propensity for IR injury in diabetic hearts is due to an enhanced susceptibility to PTP opening.

In addition to directly blocking PTP opening by targeting CyP-D, several investigators have shown that indirectly inhibiting PTP opening by reducing the ROS burden (112, 168, 185) and/or mitochondrial Ca influx (190, 195) can attenuate myocardial IR injury in non-diabetic hearts. Because the diabetic myocardium is characterized by an increased ROS burden (7), we hypothesized that the increase in oxidative stress associated with the diabetic heart may enhance the susceptibility to PTP opening and increase ischemic injury. To determine if indirectly inhibiting PTP opening by scavenging mitochondrial ROS production decreases IR injury in diabetic hearts, we used the novel cell permeable compound Bendavia (SS31 in the literature). With its dimethyl-tyrosine residue and permeability properties, Bendavia is a

mitochondria-targeted scavenger of ROS (49). In our experiments, we introduced Bendavia at the onset of reperfusion following a 20-minute period of global ischemia. Further, we show that indirect inhibition of PTP by decreasing mitochondrial Ca influx with minocycline also decreases cardiac IR injury in isolated diabetic hearts. Collectively, our data indicate that both direct and indirect inhibition of PTP opening attenuates IR injury as measured by a decrease in infarct size in isolated diabetic hearts. Like NIM811, treatment with Bendavia and minocycline reduced infarct size in diabetic hearts to the same extent as drug treated non-diabetic control hearts. These findings suggest that the increased susceptibility to IR injury in diabetic hearts is due to an enhanced susceptibility to PTP opening, mediated by an augmented ROS burden.

Redox Modulated Changes in PTP Opening

There is scant evidence to explain the mechanisms responsible for the increased susceptibility to PTP opening in diabetic hearts. Recently, two independent laboratories have demonstrated that CyP-D expression is elevated in diabetic hearts (142, 232), which could potentially explain a portion of the increased propensity for PTP opening. However, these studies did not consider potential post-translational mechanisms such as redox modifications that may alter the activity of CyP-D and lead to enhanced PTP opening. In a recent investigation, human CyP-D was shown to be redox regulated (136). In this study, Cys[203] of CyP-D exhibited redox sensitivity, in that oxidation led to decreased isomerase activity and formation of an intramolecular disulfide bridge with Cys[157]. Several studies have shown that the diabetic heart displays an oxidative shift in redox state, characterized by increased reactive oxygen species (ROS) production (32, 45, 128, 234) and a decreased capacity to buffer ROS (5, 148, 237) [also reviewed in

(41)]. Until this work, the link between oxidative stress and the enhanced sensitivity to Ca-induced PTP opening had not been established. Our data are in accordance with previous studies conducted in both humans (8) and animal models of diabetes (23, 174, 232), demonstrating that diabetic heart mitochondria have an enhanced sensitivity to PTP opening. The novelty of our work is that the augmented sensitivity to Ca-induced PTP opening displayed by isolated diabetic heart mitochondria was significantly reduced when mitochondria were treated with the reducing agent, diothiothreitol (DTT) and when animals were treated with Bendavia intraperitoneally for four days. Based on the observation that CyP-D activity can be redox modulated, it is tempting to speculate that CyP-D is more oxidized in diabetic heart mitochondria when compared to non-diabetic controls. Therefore, the potential mechanism for DTT and Bendavia-induced improvements may be due to redox modification of CyP-D. However, future investigation is needed in order to support this hypothesis.

The Ca-induced sensitivity to PTP opening in diabetic mitochondria was not completely normalized with DTT or Bendavia. One reason for this may have been that the reducing power of the DTT concentration used in our study may not have been enough to overcome the oxidized state of the redox sensitive PTP component. However, this seems unlikely due to the well known reducing capacity of DTT (74, 107, 108). Another possible explanation may be that a portion of the enhanced propensity for PTP opening in diabetic hearts is due to alterations in mitochondria-independent mechanisms, such as cell signaling pathways known to alter PTP opening [reviewed in (160)]. Although these pathways may be altered by redox state, they would not be present In our isolated mitochondrial preparation. Nevertheless, the significant

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decrease in sensitivity to PTP opening in diabetic heart mitochondria evoked by a reductive shift in redox state is a significant finding due to the fact that a reduction in the open probability of PTP has been shown to improve outcomes in patients with myocardial infarction (182).

In our study, DTT had no effect on the sensitivity to Ca-induced PTP opening in non-diabetic isolated heart mitochondria. These data are in accordance with others (66) in that treatment with a reducing agent (DTT) can not decrease the sensitivity to Ca-induced PTP opening unless conditions of oxidative stress are prevailing (63). In addition, we demonstrate that the sensitivity to Ca-induced PTP opening was highly sensitive to treatment with diamide in isolated heart mitochondria from control but not diabetic animals. Treatment with diamide enhanced the sensitivity to Ca-induced PTP opening in control animals to the same extent as non-treated and diamide treated diabetic heart mitochondria. Taken together, these data support the hypothesis the oxiditaive shift in redox state characteristic of the diabetic heart is responsible for a portion of the enhanced sensitivity to PTP opening.

Conclusions

In summary, these experiments provide evidence that the enhanced susceptibility to PTP opening in diabetic heart mitochondria explains the augmented myocardial ischemia-reperfusion injury observed. In addition, the increased propensity for PTP opening characteristic of the diabetic heart is redox-dependent, and a reductive shift in redox state decreases the Ca-induced sensitivity to PTP opening.

Chapter 3

Altered Mitochondrial Calcium Handling the Diabetic Heart

Abstract

Many diabetic patients exhibit signs of heart failure, including both systolic and diastolic dysfunction, independent of coronary artery disease or hypertension. Due to the key role of mitochondrial calcium (Ca) in supply-demand matching, impairments in mitochondrial calcium displayed by the diabetic heart have been implicated in myocardial dysfunction. The purpose of this study was to: 1) definitively determine if mitochondrial Ca influx via mitochondrial Ca uniporter (MCU) and efflux via mitochondrial Na-Ca exchanger are impaired in diabetes and 2) determine if redox-dependent changes in mitochondrial Ca transients contribute to altered mitochondrial Ca handling in diabetic hearts. Mitochondria isolated from the left ventricles of STZ-induced diabetic rats displayed slower Ca uptake when compared to non-diabetic mitochondria (P<0.05), and Na/Ca exchange activity was faster in diabetic when compared to non-diabetic (P<0.05), despite no differences in respiratory control ratio or mitochondrial membrane potential between groups. Treatment of diabetic mitochondria with 2mM of the reducing agent dithiothreitol significantly normalized MCU activity to non-diabetic levels. These findings suggest that diabetic heart mitochondria have reduced mitochondrial Ca content under physiological Ca concentrations and that the reduction in MCU activity is redox dependent. Results from this study provide insight for the potential mechanisms underlying supply-demand mismatching in diabetic hearts.

Introduction

Approximately eight percent of the US population is diabetic (84), and by the year 2025, it is estimated that the global population of diabetics will reach 300 million (125). Nearly 40 years ago, Rubler and colleagues observed manifestations of heart failure in patients suffering from diabetes, independent of coronary artery disease and hypertension (193). Specifically, these investigators observed that diabetic patients had pathological left ventricular hypertrophy and myocardial fibrosis. This heightened cardiac dysfunction observed in diabetic patients, independent of coronary artery disease or hypertension was termed *diabetic cardiomyopathy* (193). Since that time, several other manifestations of diabetic cardiomyopathy have been observed. Presently, characteristics of diabetic cardiomyopathy include: systolic and diastolic dysfunction, left ventricular hypertrophy, impaired contractile reserve and interstitial fibrosis [reviewed in (30)]. Mechanisms underlying cardiac dysfunction in diabetics are still not clear. This paucity in the literature provides a substantial barrier for the development of effective therapeutic strategies seeking to diminish heart failure in diabetic patients. Elucidating the cellular mechanisms that contribute to the augmented cardiac dysfunction in the diabetic condition may foster novel treatments for diabetic patients that experience heart failure.

Many characteristics of idiopathic diabetic cardiomyopathy overlap with those of coronary artery disease/hypertension-induced heart failure in non-diabetic patients, including both systolic (75, 193) and diastolic dysfunction (33, 198, 200) as well as metabolic inflexibility (38, 147, 226). While the pathogenesis of diabetic cardiomyopathy is likely due to a number of variables, an emerging body of evidence suggests that aberrant mitochondrial function plays a significant role in the cardiac

dysfunction observed in these patients [reviewed in (30, 41)]. Diabetic heart mitochondria exhibit impaired calcium (Ca) handling (80, 181, 215) coupled with an oxidative shift in redox state, characterized by increased reactive oxygen species (ROS) production (32, 45, 128, 234) and a decreased capacity to buffer ROS (5, 148, 237). Mitochondrial Ca is essential for cardiac function in that mitochondrial Ca serves as a nexus between myocardial supply and demand. Mitochondrial Ca fluxes are integral to ATP supply and demand matching [reviewed in (89)]. Ca serves as a key activator for several NADH producing enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (66, 99, 167). In addition to activation of NADH production, there is evidence to support that Ca directly contributes to the activity of complex V of the mitochondrial electron transport system (217). Under physiological conditions, Ca enters the mitochondria chiefly through the mitochondrial calcium uniporter (MCU) and exits through the mitochondrial Na-Ca exchanger (mNCX) (189).

Based on the bioenergetic importance of mitochondrial Ca, decrements in the activity of these mitochondrial Ca pathways may be responsible for the impaired cardiac function and decreased cardiac efficiency observed in both human (178, 179) and animal models of diabetes (31, 32). Because supply-demand mismatch is a distinguishing feature of clinical heart failure (60, 62, 166), it is logical that the impaired mitochondrial Ca handling observed in diabetes may be responsible for the cardiac dysfunction observed. To date, there has been no mechanistic insight into the alterations observed in mitochondrial Ca handling. Several investigators have shown that an oxidative shift in redox state can alter the activity of cytosolic Ca handling

proteins (65, 218). However, no laboratories have investigated the effects of the oxidative shift in redox state characteristic of the diabetic heart on mitochondrial Ca handling.

Here, we provide evidence that mitochondrial Ca influx is decreased via MCU and mitochondrial Ca efflux via mNCX is higher in isolated diabetic heart mitochondria. Further, we demonstrate that the impaired MCU activity in isolated diabetic heart mitochondria can be normalized to non-diabetic controls when treated with the reducing agent, dithiothreitol. These observations suggest that the shift in redox state characteristic of the diabetic heart impairs mitochondrial Ca handling.

Materials and Methods

All reagents used were of the highest grade commercially available (Sigma-Aldrich, United States). Calcium green 5N salt was purchased from Invitrogen (Carlsbad, CA, USA).

All animal studies were approved by the East Carolina University Institutional Animal Care and Use Committee and were in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*. 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 five 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 using a commercially available glucometer (One Touch Ultra 2, LifeScan, Milpitas, CA, USA

Cardiac mitochondria were isolated from the left ventricle of hearts utilizing a protocol similar to Boehm et al. (25). 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. Rat hearts were excised from anesthetized rats (as described above) 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 minutes 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 (1mg/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 600g for 10 minutes, and the supernatant was then centrifuged at 8000g for 15 minutes. 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 mL IS buffer. Mitochondrial protein content was determined using a BCA protein assay.

To determine the quality of our mitochondrial preparations, respiratory control ratios (RCR) 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 K_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. RCRs were calculated by dividing the state 3 respiration by the state 2 respiration.

In a method similar to that used by Kamo et al. (117), mitochondrial membrane potential ($\Delta\Psi_m$) was determined based on the activity of the lipophilic cation, tetraphenylphosphonium (TPP$^+$) using a TPP$^+$ - selective electrode and a Ag/AgCl reference electrode (World Precision Instruments, Sarasota, FL, USA). Changes in voltage were 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: $\Delta\Psi_m = 58*\log(v/V) - 58*\log(10^{F_A E/2.3RT}-1)$ (117), where ΔE, deflection in TPP$^+$ voltage from baseline; R, gas constant; T, temperature; v, mitochondrial matrix volume; V, buffer volume. The baseline voltage was taken before addition of substrate, and mitochondrial matrix volume was assumed to be of 1μL/mg protein (191). Based on a previous study (104), we assumed that matrix volumes were similar between STZ-induced diabetic rats and non-diabetic rats. For $\Delta\Psi_m$ experiments, both the TPP$^+$ - selective electrode and reference electrode were placed in a magnetically stirred chamber containing 2.5 mL mitochondria assay buffer (described above) and 0.5 mg mitochondria, supplemented with 1.2 μM TPP$^+$. Identical to RCR experiments, mitochondria were energized with 5 mM glutamate/5 mM malate. State 3 respiration was initiated with the addition of 2.5 mM ADP.

Mitochondria (0.75 mg) were suspended in mitochondria assay buffer (described above) and supplemented with the fluorescent probe 1 μM calcium green 5N salt (Ex = 506 nm, Em = 532) to track changes in extramitochondrial calcium. Fluorescence was

measured using a spectrophotometer (Photon Technology International, Birmingham, NJ, USA). Mitochondrial calcium uniporter (MCU) and sodium-calcium exchanger (mNCX) activity experiments were performed under state 2 conditions, energized with 5 mM glutamate/5 mM malate. MCU activity was measured by adding a 50 nmole pulse of $CaCl_2$ to mitochondria, and the disappearance of calcium from the extramitochondrial solution was monitored. The disappearance of calcium was quantified by calculating the tau (time to 63% of baseline fluorescence). mNCX activity was determined utilizing methods similar to those used in original works by Crompton and colleagues (55, 56). Specifically, mitochondria were loaded with 150 nmoles $CaCl_2$. Following the complete uptake of the calcium bolus and stabilization of extramitochondrial fluorescence, mNCX activity was initiated by the addition of 15 mM NaCl. To quantify mNCX activity, the area under curve was calculated for the two minutes following addition of NaCl. Two minutes was chosen because this is the time point where the mNCX-induced calcium extrusion reached a steady plateau. For experiments where redox state was manipulated, energized mitochondria were treated with either 200 μM diamide or 2 mM dithiothreitol (DTT) for the 10 minutes prior to and throughout each experimental protocol. Previous work from our laboratory has indicated that this concentration of diamide can elicit oxidative stress by significantly lowering GSH/GSSG (35). Others have shown that this concentration of DTT can act as a reducing agent and decrease the likelihood of PTP opening under oxidative conditions (63).

Data are presented as mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA or two-way ANOVA (as appropriate), with Newman-Keuls post-hoc

analysis for comparison between groups. The level of significance was established at P < 0.05.

Results

Animal characteristics*.* Rat body weights, heart weights (corrected for body weight) and fasting glucoses are presented in Table 2.1. STZ treated rats had significantly lower body weights as well as lower heart weights when compared to non-STZ treated rats (P<0.05). In order to confirm diabetes in STZ treated rats, we measured fasting blood glucose. STZ treated rats displayed significantly higher fasting glucose levels when compared to non-STZ treated rats (495 $\pm$ 21.9 vs. 114 $\pm$ 1.43, respectively; P<0.05).

Respiratory control ratio. Respiratory control ratios are presented in Figure 2.3. There were no differences in respiratory control ratios between mitochondria isolated from STZ and non-STZ treated rats (P>0.05), suggesting that the quality of mitochondrial preparation was not different between groups.

MCU and mNCX activity. Mitochondrial calcium uptake via MCU was significantly slower in isolated mitochondria from STZ treated rats when compared to non-STZ treated (Figure, 3.1; P<0.05) as measured by tau (time to 63% calcium uptake). Further, mitochondrial calcium efflux via mNCX was significantly faster in STZ treated rats (vs. non-STZ treated rats, P<0.05; Figure 3.2). The impairment in MCU activity observed in STZ treated isolated rat mitochondria was normalized to control with treatment of 2 mM dithiothreitol.

Mitochondrial membrane potential. Mitochondrial membrane potentials are presented in Figure 2.3. To rule out mitochondrial membrane potential as a potential

factor governing Ca transients, we used the TPP$^+$-selective probe. There were no differences in State 2 nor State 3 mitochondrial membrane potentials (P>0.05), suggesting no differences in the electrical driving force for Ca entry into mitochondria.

Discussion

In this study, we hypothesized that impaired mitochondrial calcium (Ca) handling displayed by the diabetic heart is the result of an oxidative shift in the redox state of diabetic heart mitochondria. The major findings of this work are 1) mitochondrial calcium influx through MCU is diminished in diabetic heart mitochondria 2) mitochondrial calcium efflux via mNCX is increased in isolated diabetic heart mitochondria 3) a reductive shift in redox state of isolated diabetic heart mitochondria normalizes mitochondrial Ca influx. To our knowledge, we are the first to show that decreased mitochondrial Ca influx through MCU in diabetic hearts can be normalized with a reductive shift in redox state.

It is widely accepted that mitochondrial Ca fluxes are integral to ATP supply and demand matching [reviewed in (89)]. Ca serves as a key activator for several NADH producing enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (66, 99, 167). Because of the essential bioenergetic role of Ca in the myocardium, alterations in mitochondrial Ca fluxes can be detrimental to normal cardiac function and efficiency. Under physiological conditions, Ca enters the mitochondria chiefly through the mitochondrial calcium uniporter (MCU) and exits through the mitochondrial Na-Ca exchanger (mNCX) [(189). Previous studies have provided evidence that mitochondrial Ca influx was slower in isolated mitochondria from diabetic hearts (80, 181, 215). Tanaka and colleagues demonstrated reduced Ca influx

into diabetic mitochondria. However, the reduced mitochondrial Ca uptake observed in diabetic hearts was coupled with a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). Because $\Delta\Psi_m$ is the primary driving force for mitochondrial Ca influx, a decrease in $\Delta\Psi_m$ will decrease Ca influx into mitochondria. Therefore, it is difficult to determine whether the difference in mitochondrial Ca influx observed in this study was solely due to decreased $\Delta\Psi_m$ or if other factors were involved, such as altered expression of MCU or mNCX. Moreover, the $\Delta\Psi_m$ measurements were determined using the lipophilic cation $TPMP^+$ in myocytes, which may have been influenced by reduced K^+ repolarizing currents observed in diabetes (133). Flarsheim and coworkers also demonstrated a reduction in mitochondrial Ca influx in diabetic rat hearts (80). However, they did not measure $\Delta\Psi_m$ in this study. In addition, these observations were made using the Ca indicator, arsenazo III, which as been shown to enhance the production of ROS (155), and it has been observed that ROS can modulate the activity of other intracellular Ca handling proteins (65, 218). Based on our findings and in light of these data, ROS induced by arsenazo III may have confounded the results of that study.

In our mitochondrial Ca transient experiments, we directly observed Ca movements utilizing the fluorescent probe Ca-Green 5N, and to our knowledge, no other laboratories have shown that MCU activity can be altered by redox state in diabetic hearts. Here, we substantiate the previous findings that mitochondrial Ca influx is slower in diabetic heart mitochondria, and this impairment is do to an oxidative shift in redox state. However, like others (221), we did not observe differences in $\Delta\Psi_m$ and can therefore rule out its electrical driving force as a causative factor for altered mitochondrial calcium transients. It should be mentioned that we cannot rule out the

possibility of MCU differential expression between diabetic and non-diabetic heart mitochondria. One potential explanation for the slower MCU activity in diabetic heart mitochondria might be due to decreased expression of MCU. Until recently, the molecular identity of the MCU has remained elusive (18, 177). In light of these new investigations, measurement of MCU expression in diabetic heart mitochondria is possible. Determination of MCU expression in diabetic heart mitochondria will provide insight into the mechanisms responsible for decreased mitochondrial Ca influx.

In addition to reduced MCU activity, we observed altered mNCX activity in mitochondria from diabetic hearts. We found that mNCX activity is increased in STZ-induced diabetic rat heart mitochondria, while Flarsheim and colleagues found no difference in activity for the same duration of diabetes (80). One reason for these differences may be due to the fact that the ROS generation caused by arsenazo III used in their study may have abolished possible differences in mNCX activity. Another potential reason for these differences may be the fact we used 15 mM NaCl (as opposed to 10 mM NaCl by Flarsheim et al.) to induce Ca efflux via mNCX, and 15 mM NaCl induces greater Ca efflux from mitochondria than 10 mM (55, 56). We chose this concentration because 15 mM NaCl has been shown to induce near maximal Ca efflux via mNCX (55) and may therefore allow us to see maximal differences. Nevertheless, under our experimental conditions, we observed that isolated mitochondria from diabetic rat hearts display decreased MCU activity and increased mNCX activity. Liu and O'Rourke observed that inhibition of mNCX with CGP-37157 can reverse decrements in NADH production in heart failure (138). Taken together, these findings suggest that the increased mNCX activity observed in diabetic mitochondria in our study may lead to

bioenergetic compromise and explain a portion of the cardiac dysfunction observed in diabetes.

From a mechanistic perspective, no investigators have explored the link between the oxidative shift in redox state characteristic of the diabetic heart and impaired mitochondrial Ca handling. Here, we demonstrate that the impaired mitochondrial Ca influx through MCU can be rescued with DTT treatment, suggesting that an oxidative shift in the redox state of diabetic heart mitochondria is responsible for the impaired MCU activity. These findings are significant because mitochondrial Ca influx is imperative for cardiac function, and improving mitochondrial influx via MCU may decrease the cardiac dysfunction observed in diabetic hearts.

Our findings suggest that under physiological [Ca], Ca is low in diabetic heart mitochondria. Extrapolating these *in vitro* findings to *in vivo* conditions, diabetic mitochondria would likely have less mitochondrial Ca. Because increasing mitochondrial Ca levels leads to an increase in ATP synthesis, while decreasing mitochondrial Ca decreases ATP synthesis (115), it is plausible that the decreased MCU activity and increased mNCX activity observed in our study explain, at least in part, the cardiac dysfunction and inefficiency observed in diabetic hearts.

Conclusions

In summary, these experiments provide evidence that mitochondrial Ca influx is slower in diabetic hearts while sodium-induced efflux of Ca is increased. Further, MCU activity is redox-dependent and impaired mitochondrial Ca influx can be normalized with a reductive shift in redox state.

Chapter 4

High Doses of Ketamine/xylazine Anesthesia Reduce Cardiac Ischemia-

reperfusion Injury in Guinea Pigs

Abstract

Choosing an appropriate anesthetic protocol that will have minimal effect on the experimental design can be difficult. Guinea pigs have highly variable responses to a variety of injectable anesthetics, including ketamine/xylazine (KX). Because of this variability, supplemental doses are often required to obtain an adequate plane of anesthesia. Our group is interested in studying the isolated guinea pig heart, and we must anesthetize guinea pigs prior to harvesting the heart. In this study, we sought to determine if larger doses of KX protected isolated guinea pig hearts against myocardial ischemia/reperfusion injury. Male Hartley guinea pigs, Crl:HA, (275-300g; n=14) were anesthetized with one of two doses of KX (Lower: K-85mg/kg, X-15 mg/kg or Higher: K-200mg/kg, X-60mg/kg). Following thoracotomy, hearts were subjected to 20 minutes of ischemia followed by 2 hours of reperfusion. The higher dose of KX significantly reduced myocardial infarct size when compared to the lower dose (36 ± 3 vs. 51 ± 6 %, respectively; $P<0.05$). Further, the higher dose of KX improved hemodynamic function as measured by increases in both left ventricular developed pressure (49 ± 4 vs. 30 ± 8 mmHg, respectively, $P<0.05$) and maximal rate of left ventricular relaxation (-876 ± 70 vs. -576 ± 120 mmHg/sec, respectively, $P<0.05$), however, the higher dose of KX did not have an impact on the maximal rate of left ventricular contraction or coronary flow. In addition, the incidence of arrhythmias was unchanged by the higher dose of KX.

These results provide evidence that supplementation of KX to ensure an adequate anesthetic plane may introduce unwanted variability in ischemia/reperfusion studies.

Introduction

In many studies using animals, measurements collected on proteins, cells, or organs, must be done following proper anesthesia, but anesthetizing animals always comes with the caveat that the anesthesia itself may be inevitably altering the behavior of the system in question. Choosing a suitable anesthesia protocol that will have minimal influence on the outcome measurements in a study while still providing an adequate plane of anesthesia, is an area of ongoing interest (209).

Among laboratory animal models used in biomedical research, guinea pigs are known to have highly variable responses to a variety of anesthetic agents, particularly those injected both intraperitoneally and intramuscularly (13, 39, 58, 81, 88, 122, 184, 208), however, the mechanism for this variation is not known. Due to the variability in responsiveness from injections, and lack of a tail for intravenous delivery, many researchers prefer halogenated inhalation anesthetics (such as isoflurane, halothane, desflurane, enflurane or sevoflurane) for adequate anesthesia in guinea pigs. While the use of volatile gas anesthetics is appropriate in many research disciplines, for investigators studying ischemia/reperfusion injury, all of these agents are contraindicated due to confounding effects of the anesthetics themselves. For example, previous studies have shown that administration of volatile anesthetics confers protection against ischemia/reperfusion injury across species in tissues including heart (53, 61, 141, 183, 206), brain (119, 211, 225), kidney (116), lung (137) and liver (20). Accordingly, many scientists interested in ischemia/reperfusion injury

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employ various injectable drug classes as anesthetics. One of the most common injectable anesthetics used for surgical procedures is a ketamine/xylazine (KX) combination, and the recommended KX dosage for guinea pigs varies considerably (30 to 120 mg/kg for ketamine and 0.2 to 13 mg/kg for xylazine (77)). This wide range likely reflects the difficulty in adequately anesthetizing guinea pigs for surgical procedures with KX (39), often necessitating supplemental injections (186).

Our group is interested in cardiac ischemia/reperfusion injury, and when compared to other rodents such as the rat or mouse, the guinea pig is preferred because the electrophysiological profile of the guinea pig heart more closely resembles larger animals (35, 201), yet guinea pigs are easier to manage than larger mammals. Because the effect of KX on cardiac function and infarction is negligible compared to other means of anesthesia (53), KX is often utilized in cardiac experiments. Consistent with other investigators (13, 58, 81, 88, 122, 184, 208), we have observed that the effectiveness of KX is highly variable among guinea pigs, and that supplemental injections of KX are often required in order to achieve an adequate surgical plane of anesthesia. In previous studies, we anecdotally noticed that guinea pigs who required supplemental KX injections also appeared to have less cardiac damage after ischemia/reperfusion. Given that we are focused on identifying novel treatments that protect the heart (34-39), variability introduced by the anesthetic regimen could significantly hinder the interpretation of our data.

In this study we sought to determine if higher KX doses used to anesthetize our guinea pigs preconditioned isolated hearts against subsequent ischemic injury. We hypothesized that hearts from animals receiving higher doses of KX would be protected

against experimental ischemia/reperfusion injury. Here, we show that higher doses of KX significantly reduce myocardial infarct size and preserve hemodynamic function when compared to lower doses of KX.

Materials and Methods

A total of 14 male Hartley guinea pigs, *Cavia porcellus*, (275-300 g, approximately 30 days old) were obtained from a commercial vendor (Charles River Laboratories, Raleigh, NC). Based on health surveillance programs performed by the vendor and research institution, the guinea pigs were free from: Sendai virus, PVM, Reo, LCMV, GAV, *Encephalitozoon cuniculi*, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *S. zooepidemicus*, *Klebsiella* sp., *Salmonella* sp., ectoparasites, and endoparasites. In order to minimize the effects of external stimuli, the following environmental conditions were maintained. Animals were socially/group housed (3 -4) per cage in a room housing only that species. The cages contained aspen bedding chips (Northeastern Products Corporation, Warrensburg, NY). All animals were allowed a minimum of 5 days to acclimate following shipping prior to their experimental use. Standard guinea pig chow (Lab Diet ProLab 5P18, St. Louis, MO) was fed *ad libitum* and all animals were offered automatic water via lixits. Environmental enrichment in the form of PVC tubes and vegetables were given to the guinea pigs. Routine husbandry care was performed by the same husbandry technician, when possible, to help familiarize the animals to routine husbandry procedures.

All research adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (169). The protocol was approved by the East Carolina University Institutional Animal Care and Use Committee and was performed in a facility accredited

by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International.

On each experimental day, animals were randomly assigned to one of two ketamine/xylazine anesthesia protocols as described in Table 3.1. The lower dose protocol used in this study was recommended by the veterinary staff and is the typical dosage of KX used for surgical anesthesia in guinea pigs among investigators at our institution. Although the lower dose protocol of KX (85/15 mg/kg) is at the higher range of dosages suggested (77), our previous experience indicated that as many as 3 supplemental doses of the lower dose protocol were sometimes needed to achieve a surgical plane of anesthesia suitable for guinea pig thoracotomy, particularly following failure to achieve anesthesia with the initial dose. For this reason and in consultation with the veterinary staff, we chose the concentrations of KX in the higher dose group (Table 3.1). Each anesthetic cocktail was administered to the guinea pigs via intraperitoneal injection. Fifteen minutes following KX injection, animals were tested for righting, toe-pinch, and corneal reflexes. Animals in the lower dose group were without righting and corneal reflexes, however, we observed a toe-pinch reflex in several animals that received the lower dose KX (which would normally warrant a supplemental injection). Therefore, because we sought to standardize the anesthetic dose for each animal to ascertain the effects on cardiac function, cervical dislocation was performed in all anesthetized animals immediately prior to thoracotomy to account for varied reflex responsiveness. Cervical dislocation was performed in guinea pigs in a manner similar to the procedure for cervical dislocation in other rodents as described in the AVMA 2007 euthanasia guidelines (1). Fifteen minutes following i.p. injection of KX, cervical

dislocation was performed on anesthetized guinea pigs by a highly trained individual under the direct supervision of the attending veterinarian and with prior approval by the East Carolina University Institutional Animal Care and Use Committee. Although cervical dislocation was performed in 275-300g guinea pigs in this study (greater than the <200g recommendation for other rodents not under anesthesia), the dislocation was performed on anesthetized animals (who had no righting or corneal reflexes) and there were no complications with any of the animals. Immediately following cervical dislocation, hearts were excised via midline thoracotomy. The aorta was secured around a cannula of a modified Langendorff apparatus (isolated perfused heart model) and retrogradely perfused (at 75 mmHg constant perfusion pressure) with buffer consisting of (in mM): 118 NaCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 4.75 KCl, 1.2 $MgSO_4$, 2.0 $CaCl_2$, and 10 glucose (equilibrated with 95/5 % O_2/CO_2), as described previously (35). A latex balloon (Harvard Apparatus Balloon size #6) was inserted through the mitral valve and into the left ventricle. Cardiac hemodynamic and electrical parameters including: left ventricular developed pressure, perfusion pressure, coronary flow rates, maximal rates of contraction and relaxation, and volume-conducted electrocardiogram, were measured constantly throughout the protocol. These parameters of cardiac function were collected and digitized using the Powerlab System (A.D. Instruments Inc, USA & Canada), and stored on a personal computer for subsequent analysis.

Following a 15 minute equilibration period, hearts were subjected to no-flow ischemia (global ischemia) for a period of 20 minutes. Following the ischemic period, flow was re-established for a period of 2 hours (reperfusion).

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At the end of the reperfusion period, hearts were cut down from the cannula, and the right ventricle and atria were removed. In order to assess the amount of tissue death, infarct size was assessed histologically as previously described (36). Briefly, the left ventricle was sliced into four slices from apex to base. Each slice was weighed and incubated in a 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 10 minutes in a slow shaking water bath (37° C). Measurement of infarct size using TTC staining is considered the gold standard for quantification of cardiac ischemia/reperfusion injury in numerous species, including dogs, rabbits, and rodents (78, 114, 228). Following the brief incubation, both sides of each slice were photographed with a digital camera associated with a dissecting microscope. Infarct areas were quantified using computer software (ImageJ software, NIH, USA). Total area at risk and infarct area were measured for each side of each slice and corrected for the wet weight of each slice. All slices were averaged, and final infarct size was expressed as a percentage of the left ventricle.

Arrhythmias were characterized as previously described (34) using the guidelines established by the Lambeth Conventions and scored using a system similar to that described by Curtis and Walker (57). Arrhythmias were scored during the reperfusion period as follows: $0 = 0 - 49$ ventricular premature beats; $1 = 50 - 499$ ventricular premature beats; $2 = > 499$ ventricular premature beats and/or 1 episode of spontaneously reverting ventricular tachycardia (VT) or ventricular fibrillation (VF) less than 60 sec in total duration; $3 = > 1$ episode of VT or VF that is < 60 sec total duration; $4 =$ reverting VT or VF or both that is < 120 s in total duration; $5 =$ VT or VT or both that is > 120 sec in combined duration; $6 =$ non-reverting (fatal) VT or VF that began

> 15 min after reperfusion; 7 = fatal VT/VF that began between 5 min and 15 min after reperfusion; 8 = fatal VT/VF that began less than 5 min after reperfusion. In addition to arrhythmia scoring, total time in VT/VF was determined throughout reperfusion.

All data are presented as mean ± SEM. A Student's t-test was used to determine differences between groups, and statistical significance was established using an alpha level of 0.05.

Results

Infarct size. Representative infarct pictures and infarct size data are presented in Figure 4.1. Following 20 minutes of ischemia and 2 hours of reperfusion, infarct size was significantly lower in hearts from animals receiving higher dose KX (n=7) when compared to the lower dose KX (n=7) treated animals (36 ± 3 vs. 51 ± 6 %, respectively; P<0.05). This reduction in infarct size suggests that KX dose-dependently influences the development of cell death.

Hemodynamics. There were no significant differences in hemodynamic parameters before the 20 minute ischemic period (Table 4.1). During this baseline period, left ventricular developed pressure, maximal rates of contraction (+dP/dt) and relaxation (-dP/dt), and coronary flow rates were similar between the higher dose and lower dose KX groups. Because normoxic hemodynamic function begins to decline in hearts after approximately 1.5 hours in our model (36), we examined hemodynamic differences between groups one hour into reperfusion. One hour into the reperfusion period, there was a significant improvement in left ventricular developed pressure and maximal rate of relaxation in animals anesthetized with higher dose KX. There were no

significant differences between higher or lower dose KX groups in maximal rate of contraction or coronary flow during the reperfusion period (Table 1; P>0.05).

Electrocardiographic changes. There were no observable differences in the incidence of reperfusion arrhythmias between higher dose and lower dose KX groups. Neither arrhythmia scores nor total time in ventricular tachycardia/fibrillation were influenced by the anesthetic regimen (data not shown).

Discussion

This study was conducted to determine if higher doses of ketamine/xylazine (KX) anesthesia influenced cardiac ischemia/reperfusion injury. The variable tolerance of guinea pigs for KX anesthesia often leads to supplemental injections before surgical procedures can be performed, yet this supplementation of anesthetic may alter the physiology of the heart. In this study, we show that higher dose KX reduces myocardial infarct size and preserves hemodynamic function in guinea pig hearts exposed to ischemia/reperfusion. To the best of our knowledge, this is the first demonstration that higher doses of KX used to anesthetize guinea pigs protect the heart against ischemic injury.

Anesthetic-induced Reduction in Infarct Size

The appropriate anesthesia protocol for animal studies is something that all laboratory animal veterinarians and investigators must take into consideration. For scientists interested in studying ischemia/reperfusion injury, this task becomes considerably more complicated. Inhalation agents are easy to administer and effective at providing anesthesia, yet are well known to confer protection against subsequent ischemia/reperfusion injury (also known as "preconditioning" the tissue against

infarction), reviewed in (61). Anesthetic-induced preconditioning (with inhalants) is observed in a variety of tissues (20, 61, 116, 119, 183, 225) and in species ranging from laboratory mice (151), rats (119, 141), guinea pigs (187), rabbits (92), dogs (152, 227) and human patients undergoing surgery (20, 116).

In cardiac ischemia/reperfusion studies, anesthesia, rather than euthanasia, is utilized to obtain a beating heart in order to avoid ischemic preconditioning, a phenomenon that has been extensively shown influence infarct size (19, 26, 239). In cardiac studies directly comparing the infarct-limiting effect of anesthetic regimens, inhalation anesthetics are the most robust in reducing infarct size when compared to injectable anesthetics (53, 224). Due to the cardioprotection afforded by halogenated anesthetics, injectable anesthetics such as KX, pentobarbital or propofol are most frequently used in ischemia/reperfusion studies. Among these injectable regimens, KX anesthesia exerts a smaller influence on infarct size than propofol or pentobarbital (53, 92, 224). Recent recommendations against the use of pentobarbital due to a narrow safety window and questions of efficacy (40, 203, 233) provide further support for KX anesthesia. Lending additional support for the use of KX anesthesia, propofol is given intravenously and therefore can be difficult to administer in guinea pigs due to the difficulty associated with gaining IV access.

While KX appears to be an appropriate anesthetic regimen for ischemia/reperfusion studies, the difficulty attaining an adequate plane of anesthesia, especially in the guinea pig, frequently necessitates anesthetic supplementation (often multiple times) (39). Although supplementing the anesthetic is very common (and often necessary to achieve an adequate surgical plane), the authors are not aware of any

reports where increasing the anesthetic dose was shown to alter ischemia/reperfusion injury. In this study, we compared a higher dose to a lower dose of KX in the guinea pig and found that the higher KX dose reduced infarct size by approximately 30%, indicating that supplemental anesthetic KX doses may be introducing variability into experimental data by disproportionally preconditioning the tissue.

Effect of KX Anesthesia on Cardiac Electrical/mechanical Function

In addition to examining the effect of varying KX doses on infarct size, we also examined the influence of higher dose KX on cardiac function. The absence of baseline differences in left ventricular function between higher and lower KX doses (Table 1) is consistent with previous reports showing that KX anesthesia had minimal effects on baseline pressure development (53). This lack of influence on baseline cardiac function also provides support for KX use in cardiac functional studies given that volatile anesthetics are all potent negative inotropes (51-53, 71, 72, 118, 143, 149, 170, 171, 194, 227, 229).

Although different types of anesthetic drugs have been shown to influence recovery and incidence of arrhythmia (14, 156, 164, 235), we are not aware of studies that have examined the influence on post-ischemic functional recovery with a specific injectable anesthetic regimen. Following ischemia, higher dose KX anesthesia preserved the pumping ability of the left ventricle (Table 3.1). To the best of our knowledge, the finding that higher doses of KX influence the mechanical recovery of the heart during reperfusion is novel, and underscores the need for standardization of anesthetic agents (discussed below). The dose of KX used in anesthesia did not influence the propensity for ventricular arrhythmia in our study. These findings are also

notable, as investigators who are primarily interested in cardiac electrophysiology may not need to be as concerned about the influence of KX anesthesia on propensity for arrhythmia.

Effect of KX Anesthesia on Coronary Flow

In order to determine if differences in coronary perfusion could account for the cardioprotection we observed with high KX, we measured coronary flow rates from hearts in the study. Given that the xylazine is an agonist to α-2 adrenergic receptors, we postulated that the higher KX regimen may be constricting the coronary arteries and leading to hypoxic/ischemic preconditioning of the myocardium. Higher doses of KX did not alter the coronary flow rate during the baseline period or reperfusion, indicating that the cardioprotection with higher KX doses is independent of altered cardiac perfusion.

Standardization of the Anesthetic Regimen

In terminal surgery studies where supplemental KX could confound the outcome variables, we propose an alternative means for tissue harvest. The combination of KX followed by a physical method of euthanasia by appropriately trained individuals in anesthetized animals (K: 85 mg/kg and X: 15 mg/kg, as described in the Materials and Methods) successfully standardizes the dose of anesthetic while ensuring animals are rendered insensate prior to thoracotomy and heart removal. It is important to note that this alternative is only applicable to terminal procedures where euthanasia is acceptable with regard to the experimental conditions. In survival or *in vivo* studies where procedures are performed on anesthetized animals, the necessary supplementation to maintain a surgical plane of anesthesia may be unavoidable. In this case, the need to document the amount of anesthetic administered to each animal is of primary

importance, and potential variability introduced by anesthetic supplementation must be acknowledged as a limitation to the experimental design.

Although our study examined the effect of a higher KX dose on cardiac ischemia/reperfusion injury, we should note that higher doses of KX anesthesia may also alter infarct size in other tissues exposed to ischemia/reperfusion. Similar to cardiac tissue, brain, liver, and kidney tissues may also be affected by higher doses of KX. We suspect that like cardiac tissue, infarct size following ischemia may also be reduced in these tissues when supplemental KX doses are used during anesthesia, but future studies must be conducted to support this notion.

Potential Mechanisms for Cardioprotection by Ketamine/Xylazine

In our study, we increased the dosage of both ketamine and xylazine and determined that in combination, KX acts as a cardioprotectant following ischemia/reperfusion injury. While we cannot decipher the individual effects of higher ketamine or xylazine doses on myocardial ischemia/reperfusion injury, we will briefly speculate on mechanisms of action for each compound. Ketamine is a dissociative anesthetic that exerts its inhibitory actions by deactivating the N-methyl d-aspartate receptor (NMDAR), a non-selective cation channel. We are not aware of any studies directly examining the cardioprotective effects of ketamine, but ethanol, another NMDAR antagonist, has been shown to have cardioprotective properties (121, 175, 212). It is possible that increased glutamate release after NMDAR inhibition, which preconditions other tissues against cell death (196), was responsible for the reduction in ischemia/reperfusion injury observed in the higher dose KX group. Ketamine equilibrates into tissues rapidly, with a serum half-life of 13 minutes in rodents following

ip injection (150). We cannot rule out the possibility that higher ketamine groups had greater heart uptake of ketamine, and that the protection observed may have involved higher tissue levels of ketamine. However, a previous study administered ketamine immediately before ischemia and found that it had no effect on infarct size (158), arguing against a correlation between tissue ketamine levels and protection from infarction.

Xylazine is both an analgesic and sedative, and has been shown to be an α_2-adrenergic agonist (44). Stimulation of α_2-adrenergic receptors in heart leads to vasoconstriction, which might result in ischemic preconditioning of the tissue. However, we saw no differences in baseline coronary flow rates between higher and lower KX doses, which suggests that any vasoconstricting properties of xylazine must be minimal and/or short-lived. Based on previous reports where increasing the xylazine content dose-dependently influenced cardiac function (236), and in other studies where treating with ketamine alone immediately prior to ischemia did not alter infarct size (158), it is tempting to speculate that increased doses of xylazine may be responsible for the cardioprotection observed in this study. Future studies are needed to determine if increases in ketamine or xylazine alone contribute to cardioprotection, or if an increased combination of the two is necessary for cardioprotection (as observed herein).

Study Limitations

One limitation to our study is that there is no true control group, as all animals received KX prior to harvesting the heart. While we cannot determine the extent of protection induced by the low-dose KX group compared to a "no-drug" group, we chose not to perform cervical dislocation on un-anesthetized animals to be in accordance with

AVMA guidelines on rodents greater than 200g (1). This limitation is also found in the literature, where studies comparing different anesthetics also have no true control group (53, 92, 158). Among the studies contrasting different anesthetics, the magnitude of infarct-size reduction following KX anesthesia is modest, especially when comparing to volatile anesthetics that exert a much more profound influence on infarct size (53, 92).

Furthermore, our study was specifically designed to test two distinct concentrations of KX. Anesthetic supplementation with small incremental doses may not sufficiently precondition the heart as much as the wider range used herein, but it is clear that large differences in KX doses can substantially influence the susceptibility of the heart to injury. Awareness of the potentially confounding effects of anesthetic supplementation, especially in animals where an adequate plane of anesthesia is difficult to achieve, should help investigators with the most accurate interpretation of their data. It is not our purpose to suggest the use of the high dose protocol for anesthetizing guinea pigs, however, we want to emphasize the fact that the use of additional doses of KX can confound the interpretation of data obtained in cardiac ischemia/reperfusion studies.

Conclusions

In this study we found that higher doses of KX used to anesthetize guinea pigs led to a reduction in myocardial infarct size and led to improved hemodynamic function after experimental ischemia/reperfusion. In studies examining ischemic injury, significant supplementation of KX to ensure adequate anesthesia in guinea pigs may be introducing unwanted variability.

Chapter 5

Integrated Discussion

This work has provided novel insight into the mechanisms underlying cardiac dysfunction and the enhanced susceptibility to death following myocardial infarction in diabetic patients. In addition, this work has demonstrated that the anesthetic cocktail, ketamine-xylazine, can precondition the heart and provide protection against ischemia-reperfusion (IR) injury. This report provides support for the hypothesis that the augmented ischemic injury observed in diabetic patients is due to an enhanced propensity for mitochondrial permeability transition pore (PTP) opening. Support for this hypothesis lies in the fact that direct and indirect inhibition of PTP opening at the onset of reperfusion following 20 minutes of ischemia in isolated diabetic rat hearts resulted in a significant decrease in infarct size. Infarct size was reduced to the same extent in diabetic hearts when compared to non-diabetic hearts, suggesting that enhanced PTP opening is the primary factor governing augmented injury in the diabetic heart. Perhaps of most clinical relevance, we introduced the use of three novel mitochondria-targeted therapies to achieve reduction in infarct size. Because mitochondria appear to be one of the primary entities governing IR injury through induction of PTP, the ability of NIM811, Bendavia and minocycline to target mitochondria and inhibit PTP opening is ideal.

Coupled with an enhanced susceptibility to ischemic injury, diabetic patients exhibit several signs of heart failure, including: systolic and diastolic dysfunction, left ventricular hypertrophy and interstitial fibrosis, independent of coronary artery disease

or hypertension. An emerging body of evidence has indicated that aberrant calcium (Ca) handling may be responsible for the manifestations of heart failure present in many diabetic individuals. Calcium handling is required for the mechanical work performed by the heart as well as the mitochondrial ATP producing processes that support contraction. In order to adequately support energy production with demand, mitochondrial Ca levels must be coordinated. Ca enters the mitochondria down its electrochemical gradient through the mitochondrial Ca uniporter (MCU) and exits the mitochondria in a sodium-dependent manner via the Na-Ca exchanger (mNCX). This work has illustrated that mitochondrial Ca handling is impaired in diabetic hearts. Specifically, mitochondrial Ca influx was decreased, while sodium-induced Ca efflux was increased. These findings suggest that mitochondrial Ca may be low in diabetic hearts under physiological [Ca]. These findings compliment those of a recent study where inhibition of mNCX activity rescued heart failure (139). Because a decrease in mitochondrial Ca can lead to bioenergetic compromise, the results of this work indicate that the cardiac dysfunction observed in diabetic patients may be due to impaired mitochondrial Ca handling, similar to that seen heart failure.

Taken together, the findings that diabetic heart mitochondria are able to retain less Ca via the activities of MCU and mNCX in the presence of enhanced sensitivity to Ca-induced PTP opening should not be misinterpreted. To reference Dr. Andrew Halestrap, like Dr. Jekyll and Mr. Hyde, mitochondria possess two distinct personas: under physiological conditions, mitochondria utilize Ca to maintain ATP production in order to meet the cellular demand. However, when overloaded with Ca, mitochondria summon their dark side and may induce necrotic and/or apoptotic cell death. The

distinct role of mitochondria during physiological and pathological (i.e. ischemia-reperfusion) conditions underscores the importance for careful regulation of mitochondrial Ca. From a Teleological perspective, it is interesting to speculate that in the diabetic heart, mitochondria may be down regulating mitochondrial Ca levels in an attempt to decrease the likelihood of PTP opening and avoid cellular injury, since mitochondrial Ca overload is a significant contributor to PTP induction.

This work demonstrated that blocking mitochondrial Ca at the onset of reperfusion provided significant protection in both non-diabetic and diabetic isolated rat hearts subjected to a 20-minute ischemic insult. While the acute use of mitochondrial Ca blockers may be a beneficial treatment during times of Ca overload in diabetic hearts, drugs such as minocycline may be detrimental to normal cardiac function. The reduction in mitochondrial Ca observed in diabetic hearts may be further exacerbated with the use of minocycline. Therefore, chronic use of minoclycline and/or other compounds that decrease mitochondrial calcium may need to be avoided in diabetic populations or other conditions characterized by bioenergetic compromise such as patients with heart failure.

Chapter 4 of this work was ancillary to the main body, however, it is significant in that ketamine-xylazine (KX) can precondition the heart and protect against IR injury. The implications of this study are that continuous supplementation of KX anesthesia often required in guinea pigs may introduce confounding results in IR injury studies. While we did not provide mechanistic insight into the cardioprotective properties of KX in our experiments, a recent study by Chang and colleagues revealed that ketamine can depress cytosolic Ca levels in hepatocytes (47) and therefore may be able to decrease

intracellular Ca overload. However, the impact of ketamine on mitochondrial Ca was not revealed. Based on the previously described relationship of cytosolic and mitochondrial Ca, these observations provide support for the hypothesis that that ketamine may decrease the likelihood of PTP opening by reducing cellular and mitochondrial overload of Ca.

Future directions

In this work, we demonstrated that diabetic hearts are more susceptible to IR injury, and this increased propensity for IR injury was due to enhanced PTP opening. Further, PTP opening in isolated mitochondria from diabetic hearts was shown to be redox-modulated. Because CyP-D has been shown to be redox-modulated, it would be interesting to determine if CyP-D is more oxidized in diabetic heart mitochondria when compared to non-diabetic and if this shift in redox state is associated with the enhanced propensity for PTP opening in diabetic hearts. This determination could be made utilizing the redox western blotting technique and could provide insight into a potential therapeutic target in diabetic patients. It would also be worthwhile to determine if MCU and mNCX are oxidized in diabetic hearts. The present data would suggest that MCU in diabetic hearts is characterized by an oxidative shift in redox state. Further, manipulation of mitochondrial Ca in isolated diabetic hearts exposed to varying workloads may provide insight into how mitochondrial Ca handling alters cardiac function in diabetes. For instance, increasing mitochondrial Ca influx with spermine or decreasing mitochondrial efflux utilizing CGP-37157 may improve cardiac function in diabetic hearts. While this work has provided many insights into the mechanisms underlying enhanced injury and cardiac dysfunction in diabetes, future investigation is

needed in order to gain additional insight and promote the development of therapeutic

strategies seeking to mitigate these pathologies.

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Figure 1.1 Time course of intracellular changes in rat myocardium during ischemia; $\Delta\Psi_m$, mitochondrial membrane potential; $\Delta\Psi_p$, sarcolemmal membrane potential; SarcK$_{ATP}$, sarcolemmal ATP-sensitive potassium channels; PTP, mitochondrial permeability transition pore; *adapted from Frasier et al., 2011.*
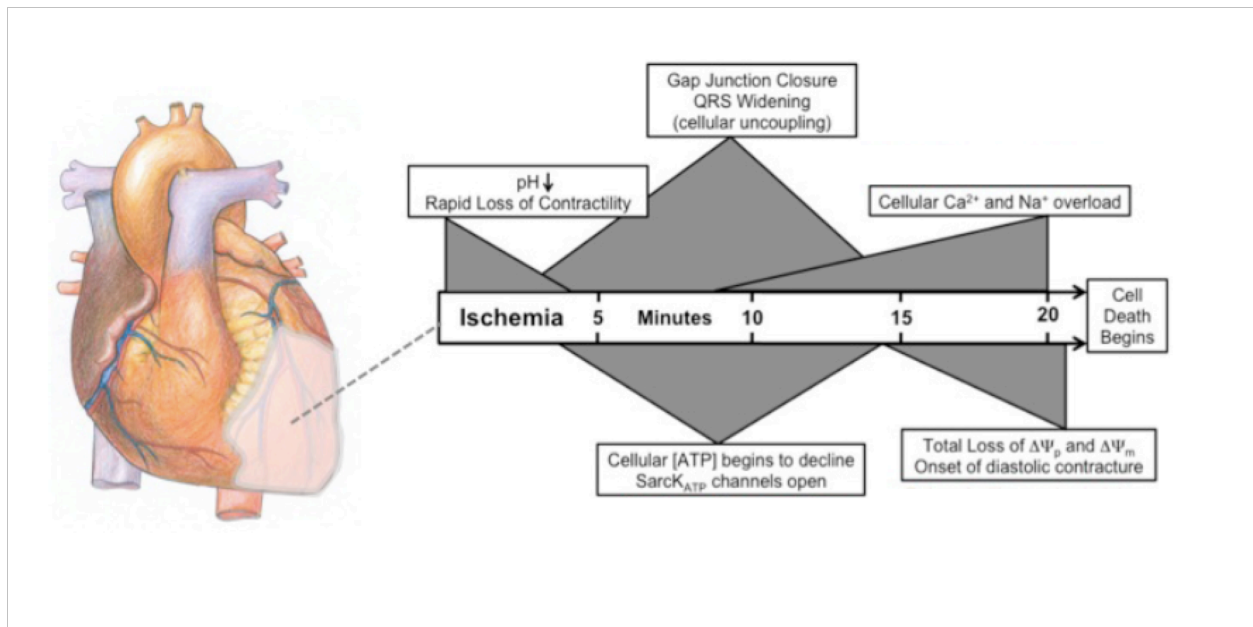
Figure 1.2 Schematic of inner mitochondrial membrane depicting putative components of the permeability transition pore during normoxia; IMM, inner mitochondrial membrane; ANT, adenine nucleotide transferase; PiC, mitochondrial phosphate carrier; CyP-D, cyclophilin D; *figure from Brown and O'Rourke, 2011.*
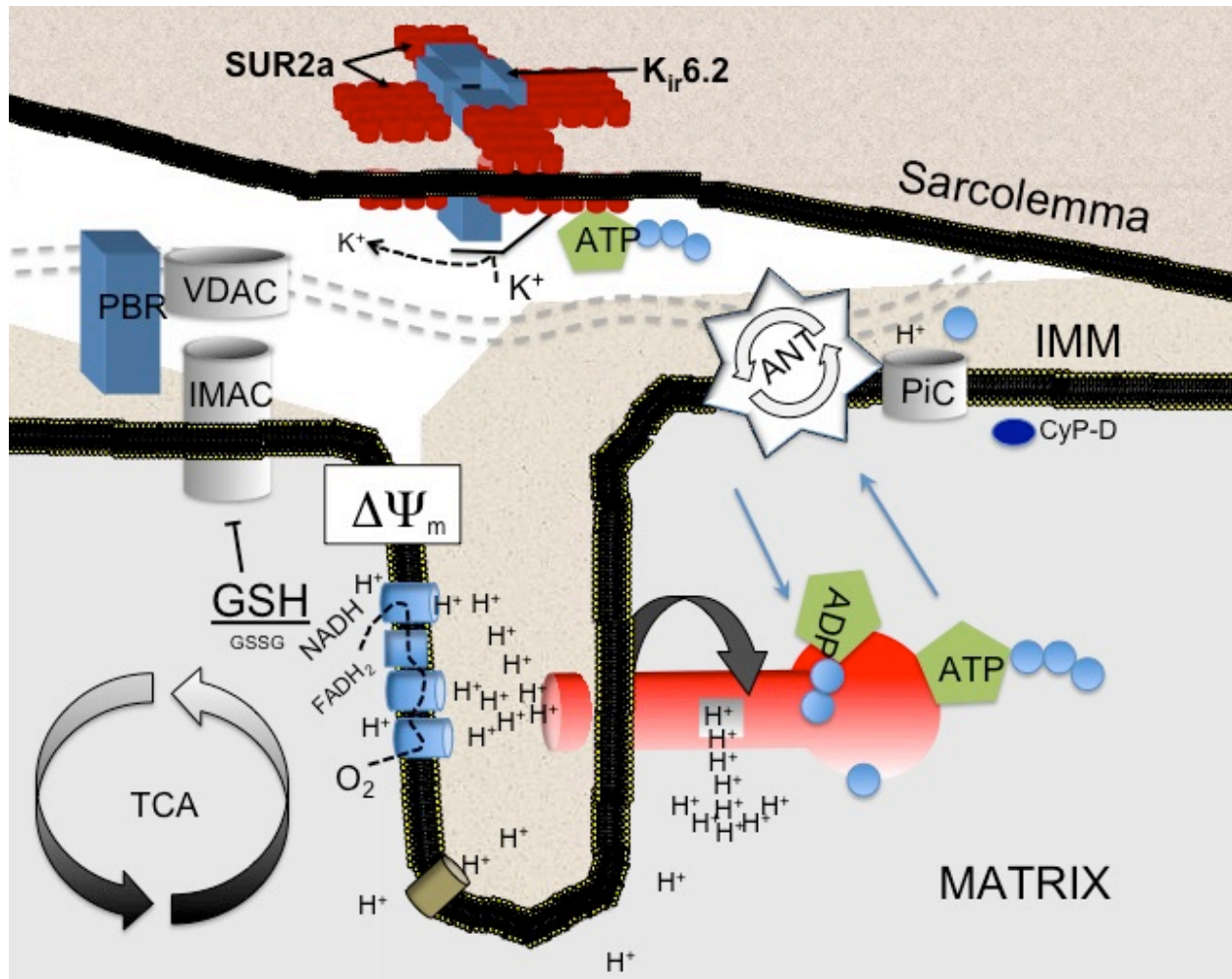
Figure 1.3 Confirmation in isolated mitochondria that minocycline is just as effective as Ru360 at blocking mitochondrial calcium uptake via MCU; Ca, calcium; MCU, mitochondrial calcium uniporter.
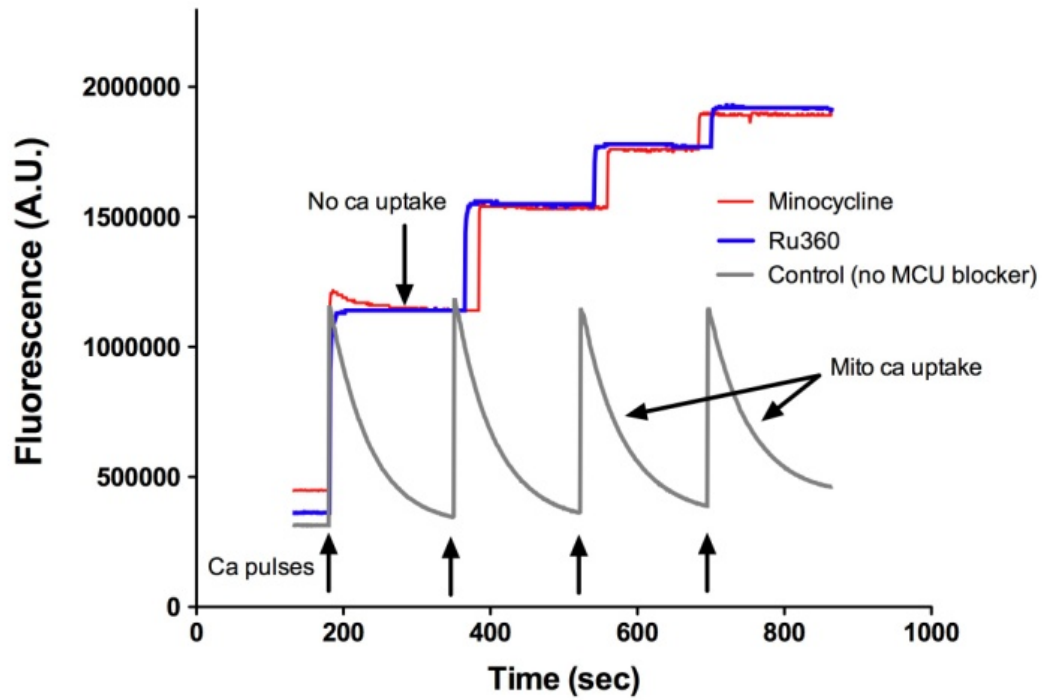
Figure 1.4 Effect of Bendavia on infarct size in guinea pig hearts subjected to 20 minutes of ischemia followed by 120 minutes of reperfusion.   Data are presented as mean $\pm$ SEM. *, P<0.05 versus non-drug treated control.
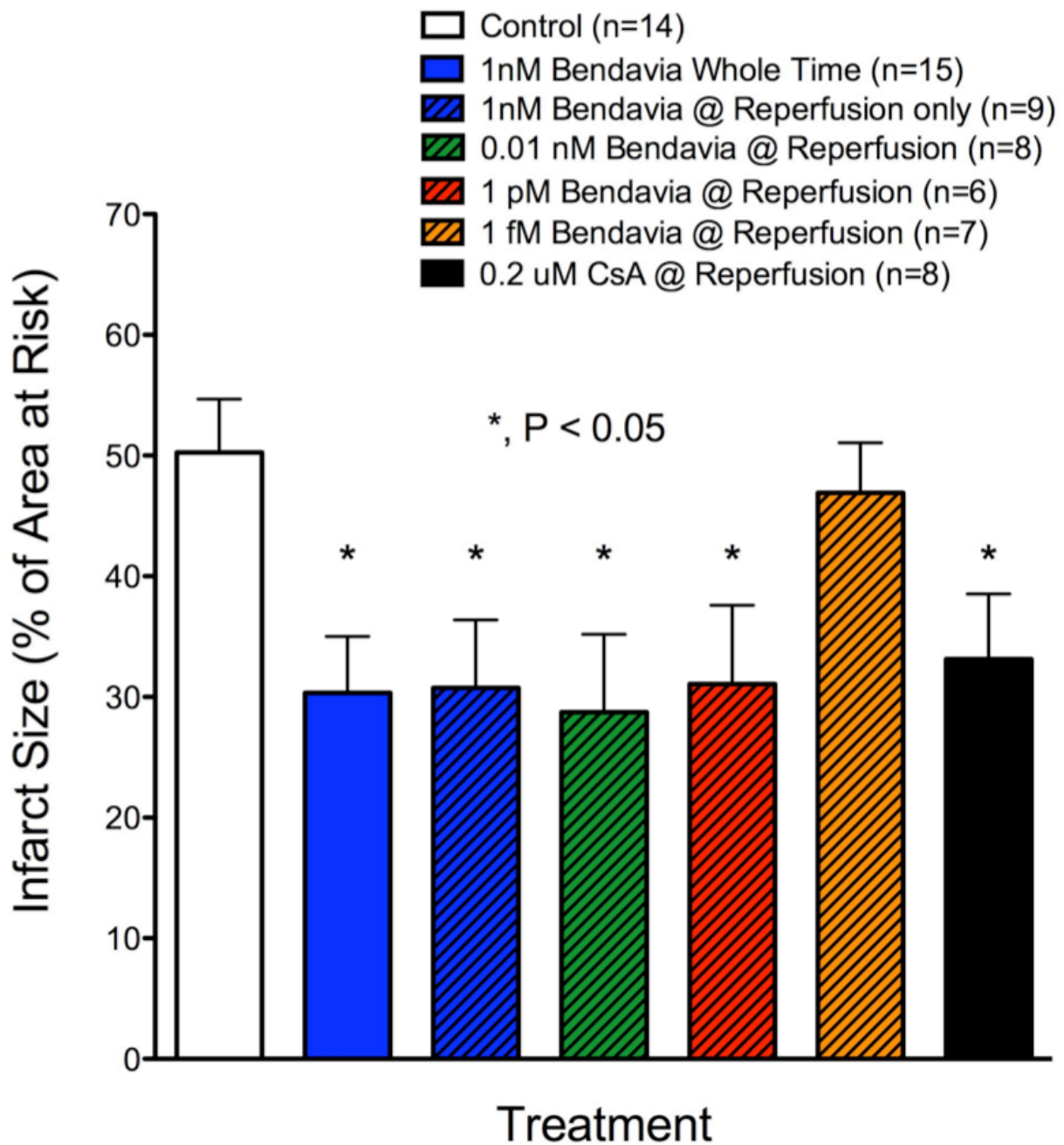
Figure 1.5 Schematic depiction of cytosolic and mitochondrial calcium transients in myocardium. SR, sarcoplasmic reticulum; RyR, ryanodine receptor; SERCA, ATP-dependent sarco/endoplasmic reticulum Ca-ATPase; MCU, mitochondrial calcium uniporter; mNCX, mitochondrial sodium-calcium exchanger.
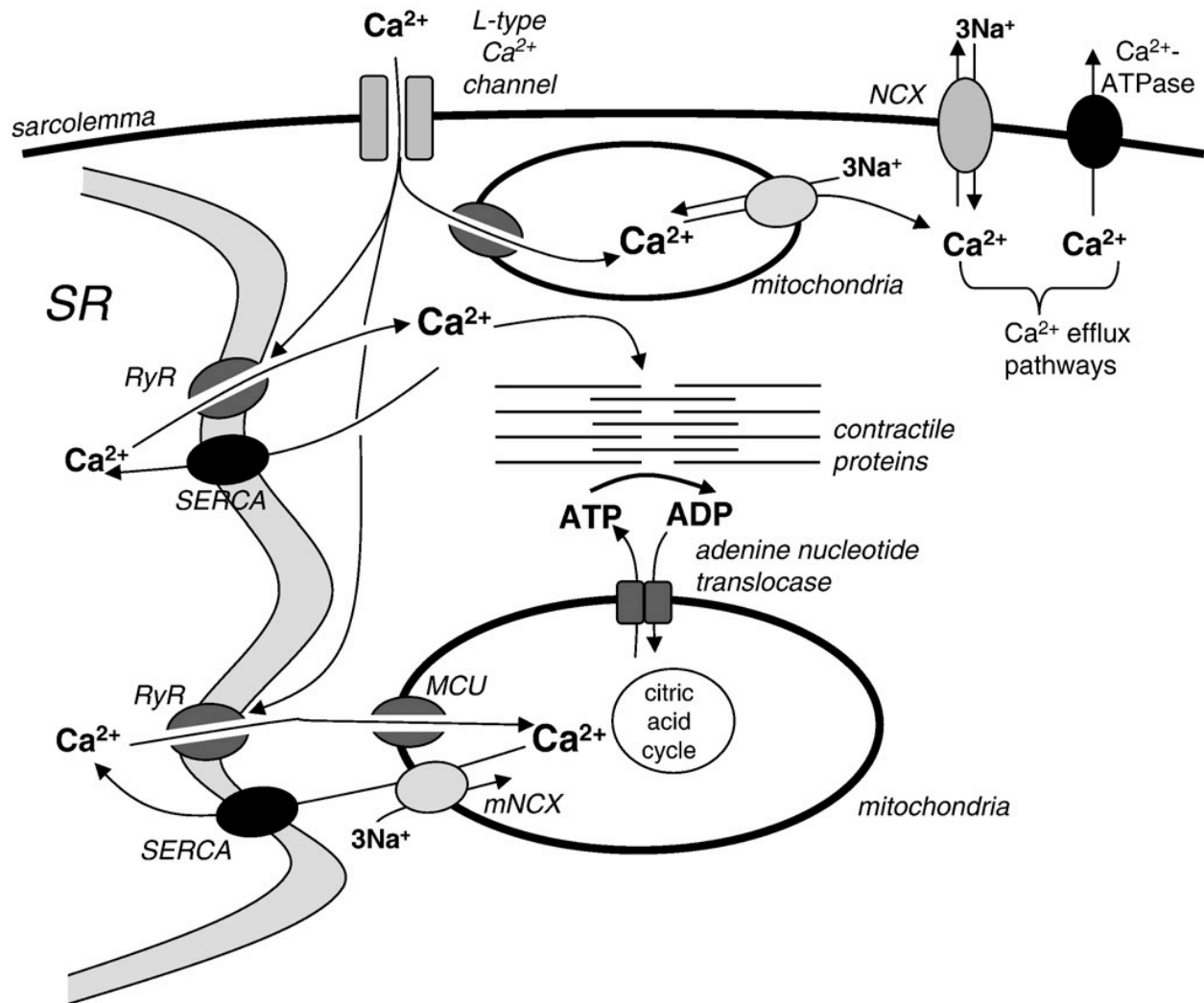
Figure 1.6 Schematic depiction of the mitochondrial calcium circuit. IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; MCU, mitochondrial calcium uniporter; mNCX, mitochondrial sodium-calcium exchanger; PTP, permeability transition pore; MTP-131, Bendavia; *figure courtesy of Dr. David A. Brown.*
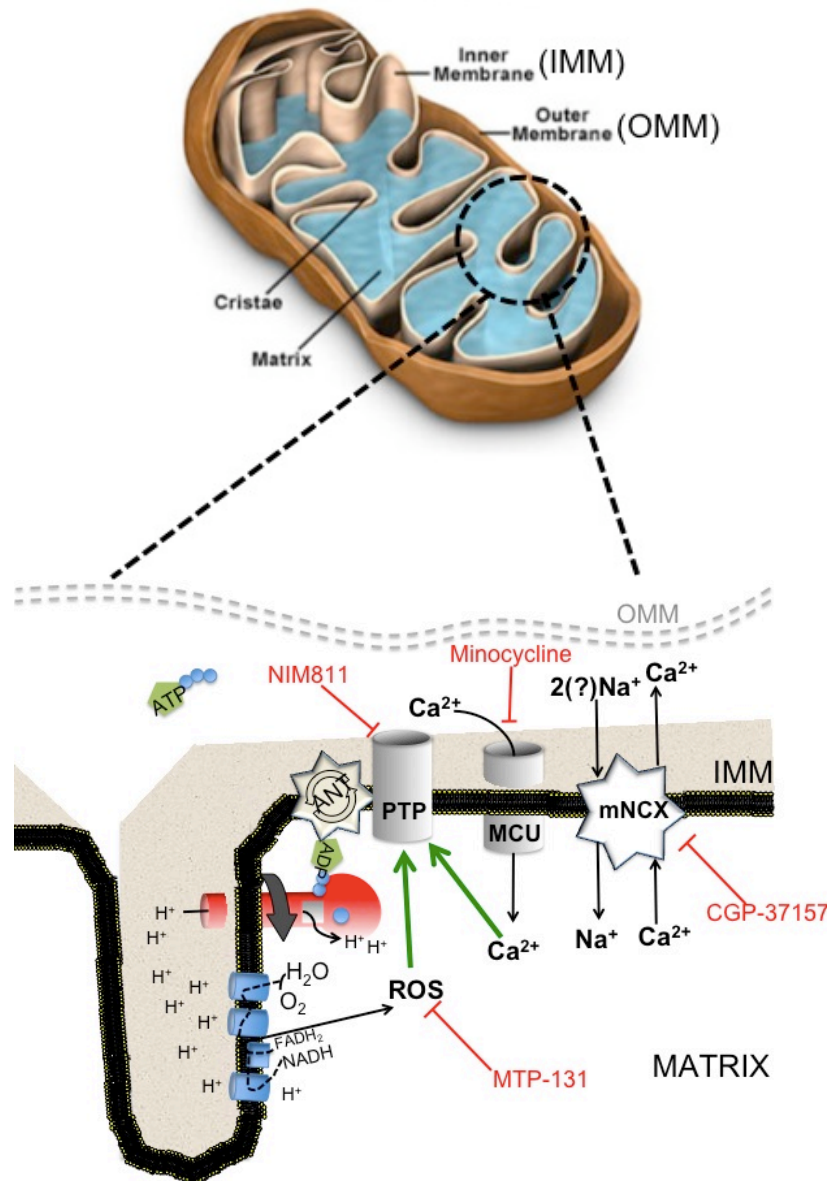
Figure 2.1 Representative infarct pictures and quantification of infarct sizes. TTC stained viable tissue bright red, while infarcted tissue appears pale in color. Upper left, control; upper right, STZ; lower left STZ+NIM811; lower middle, STZ+Bendavia; lower right, STZ+minocycline; Control, non-diabetic rats; STZ, diabetic rats; data are presented as mean $\pm$ SEM; *, P<0.05 versus non-drug treated control; #, P<0.05 versus non-drug treated STZ; N=5-10 in each group.
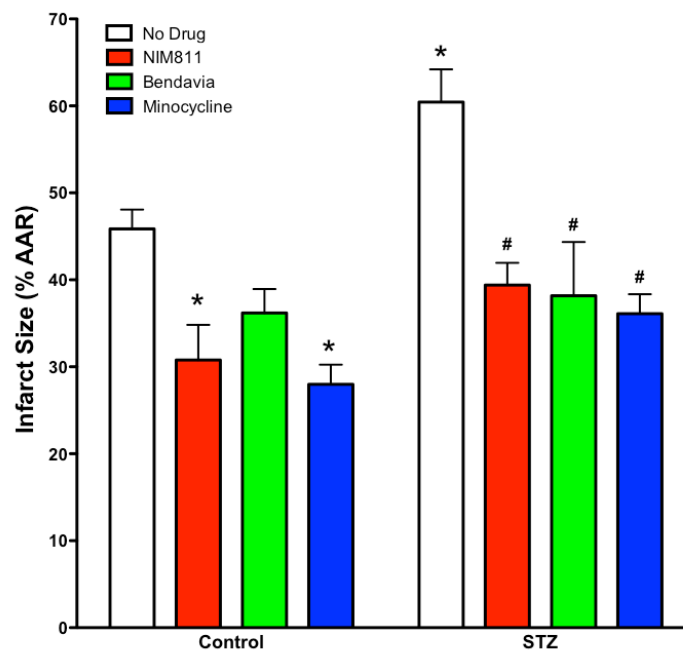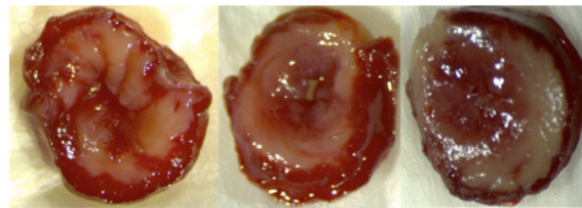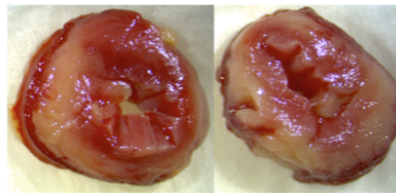
Figure 2.2 Representative trace of transition from normal sinus rhythm to ventricular fibrillation, as well as quantification of arrhythmia scores from isolated rat hearts following 20 minutes of ischemia and 120 minutes of reperfusion. VF, ventricular fibrillation; Control, non-diabetic rats; STZ, diabetic rats; data are presented as mean $\pm$ SEM. There were no significant differences between groups, N=5-10 in each group.
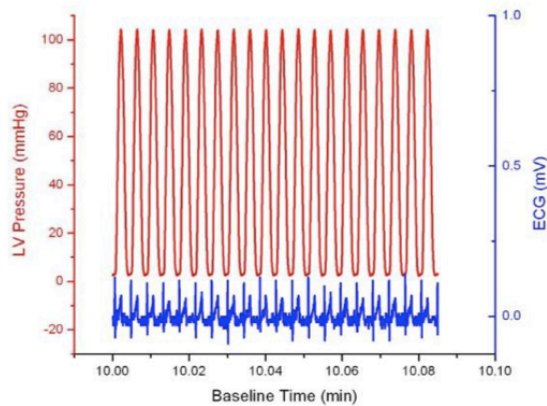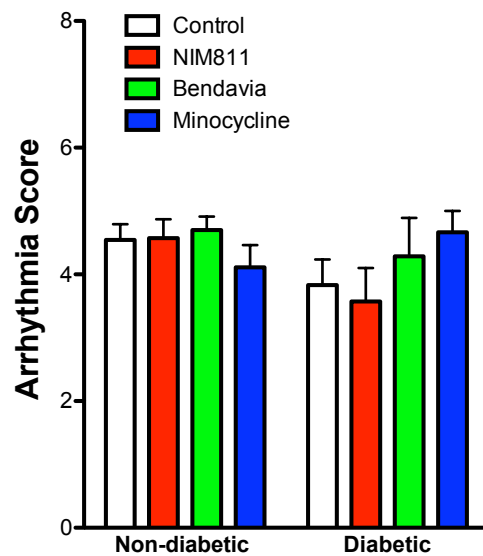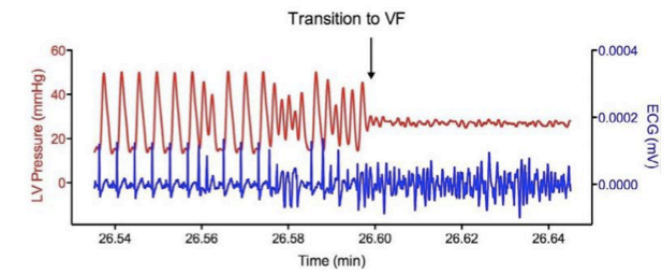
Figure 2.3 Representative trace and quantification (next page) of mitochondrial membrane potential and respiratory control ratio from isolated heart mitochondria; there were no differences in State 2 or State 3 membrane potentials or respiratory control ratios between STZ and control rats; $P > 0.05$; Control, non-diabetic; STZ, diabetic.
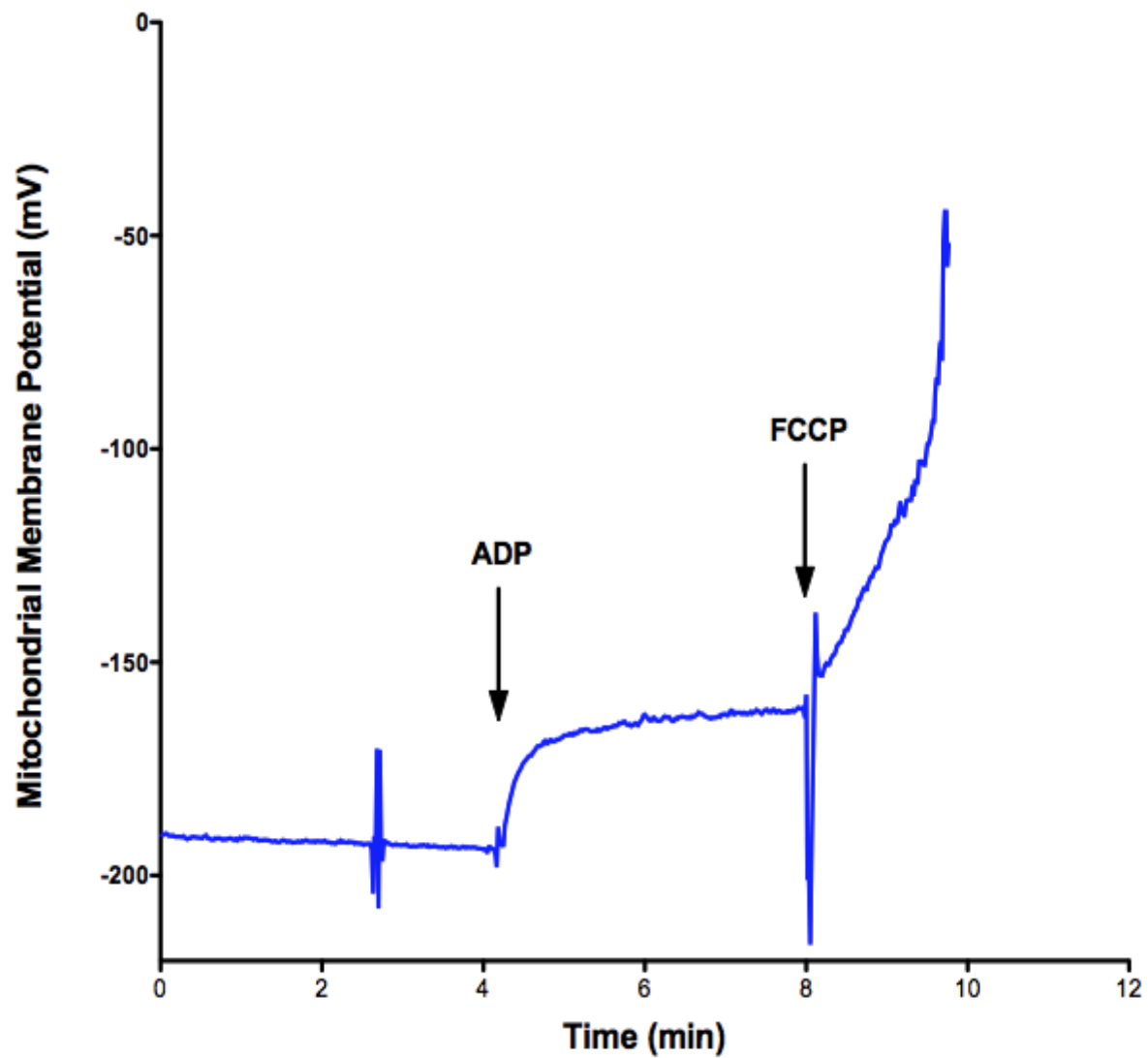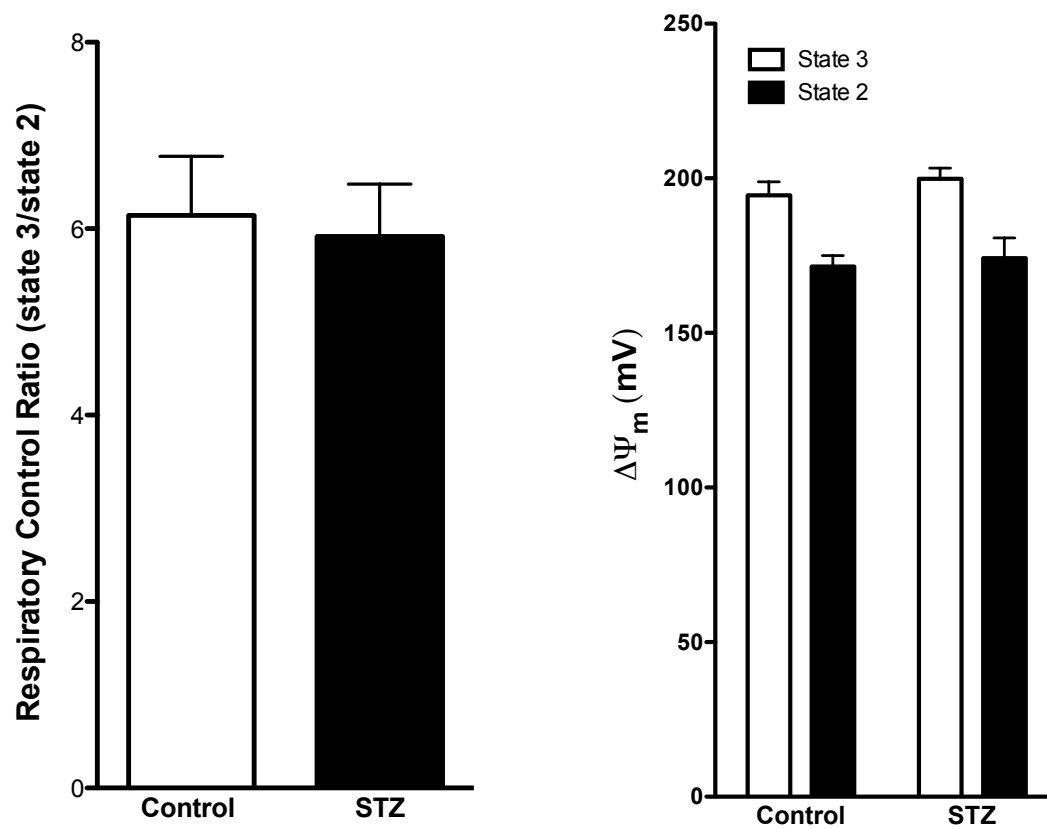
Figure 2.3 continued

Figure 2.4 Representative fluorescence trace of Ca-induced PTP opening in STZ versus STZ+DTT isolated heart mitochondria as well as quantification (next page) of calcium retention capacity in isolated mitochondria. Control, non-diabetic; STZ, diabetic; DTT, dithiothreitol; PTP, permeability transition pore; *, P<0.05 versus non-drug treated control; #, P<0.05 versus non-drug treated STZ; N=7-10 in each group
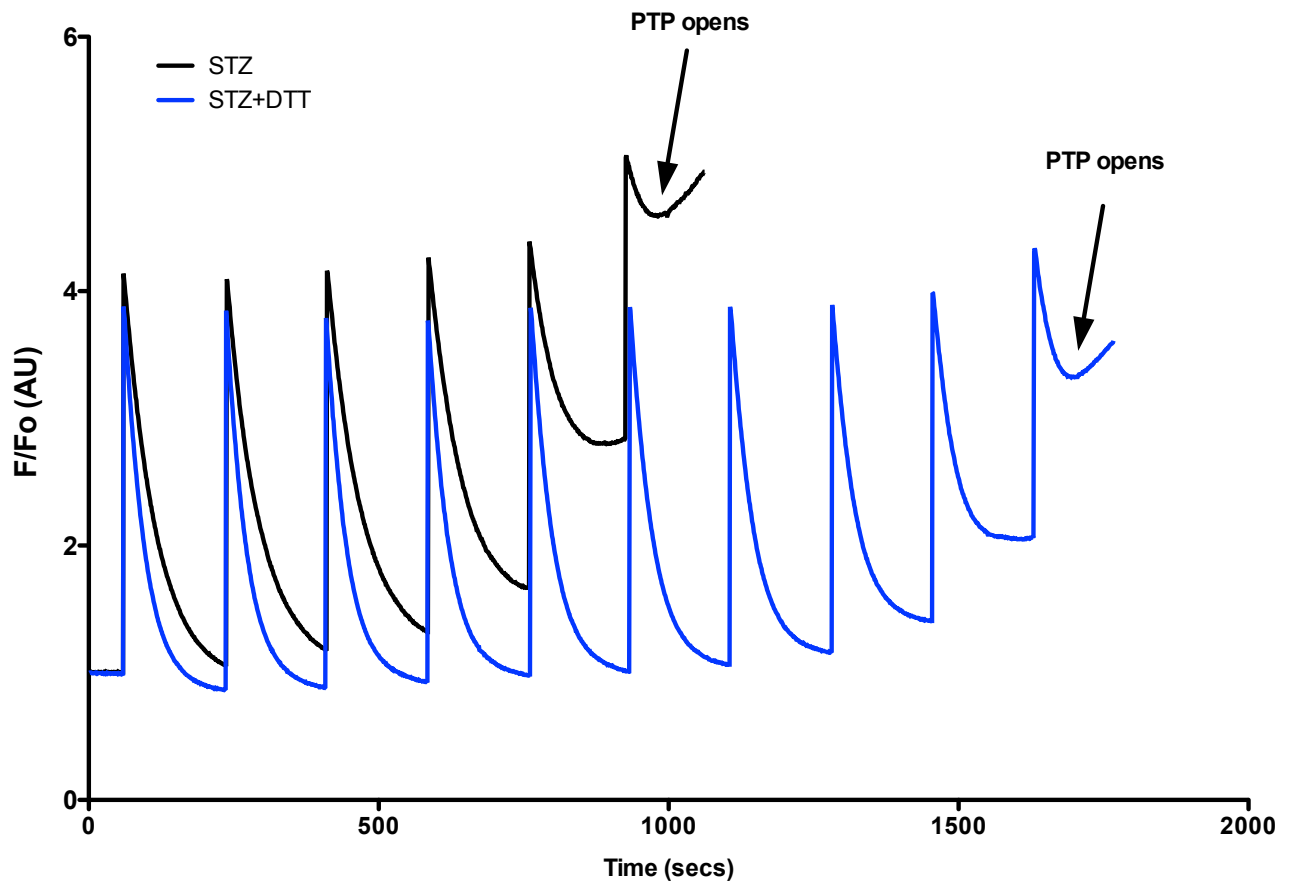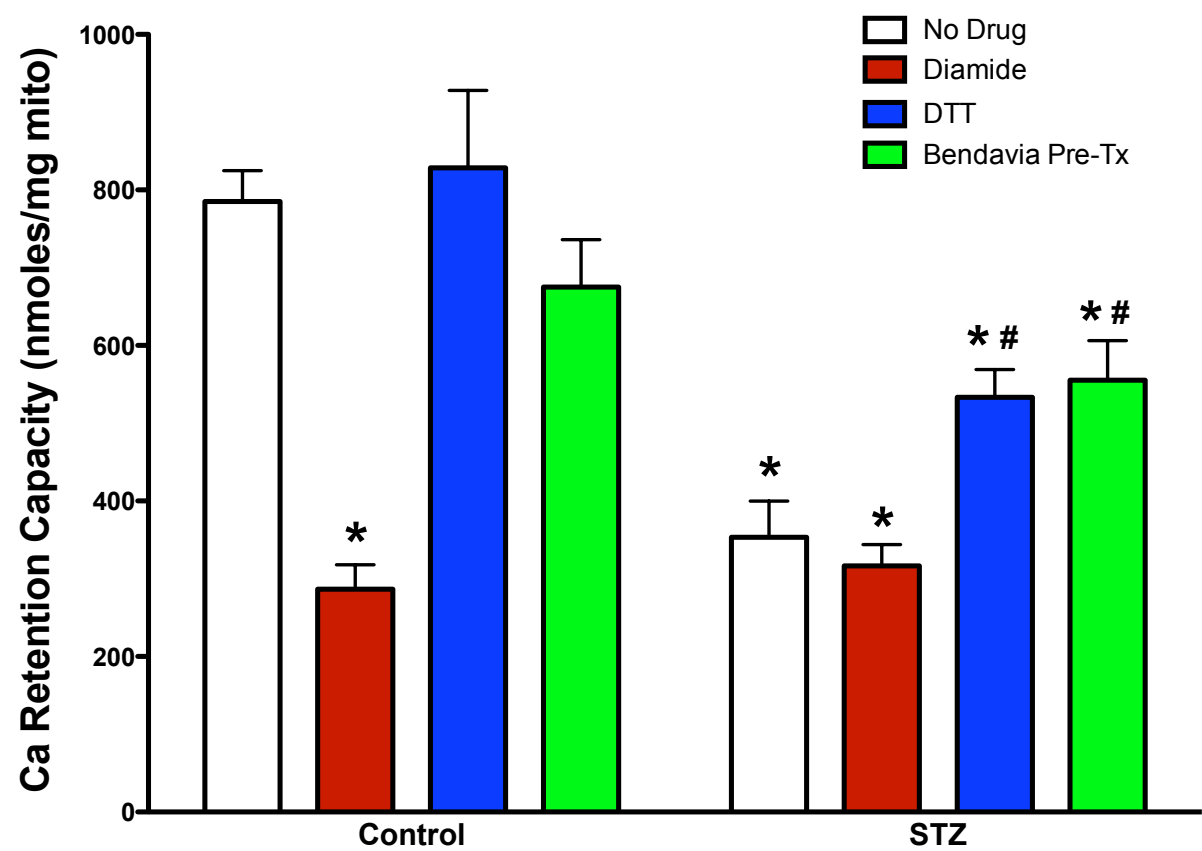
Figure 2.4 continued

Figure 3.1 Representative trace and quantification of mitochondrial calcium uptake; Control, non-diabetic; STZ, diabetic; DTT, dithiothreitol; *, P<0.05 versus non-drug treated control, N=7-10 in each group.
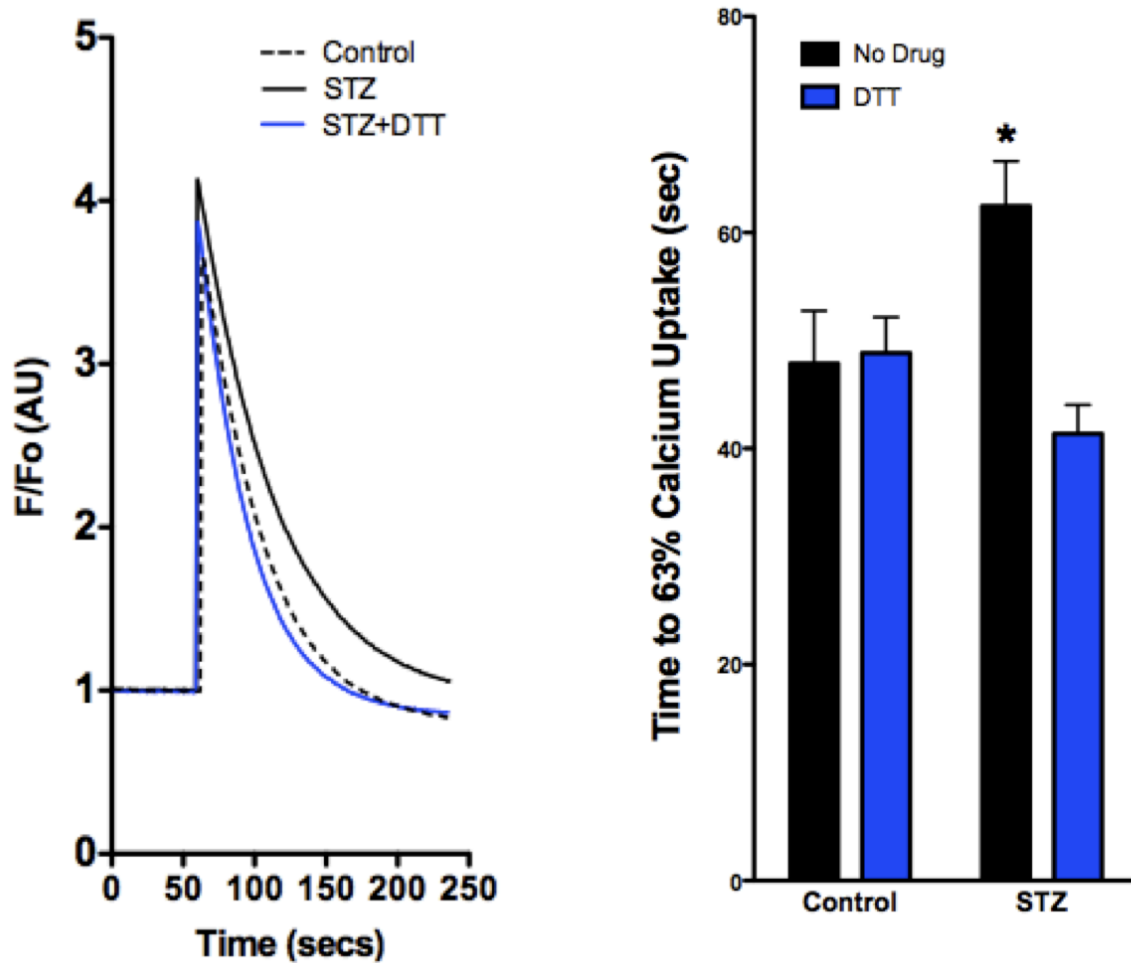
Figure 3.2 Representative trace and quantification of mitochondrial sodium-induced calcium efflux; Control, non-diabetic; STZ, diabetic; *, P<0.05 versus control, N=9-12 in each group.
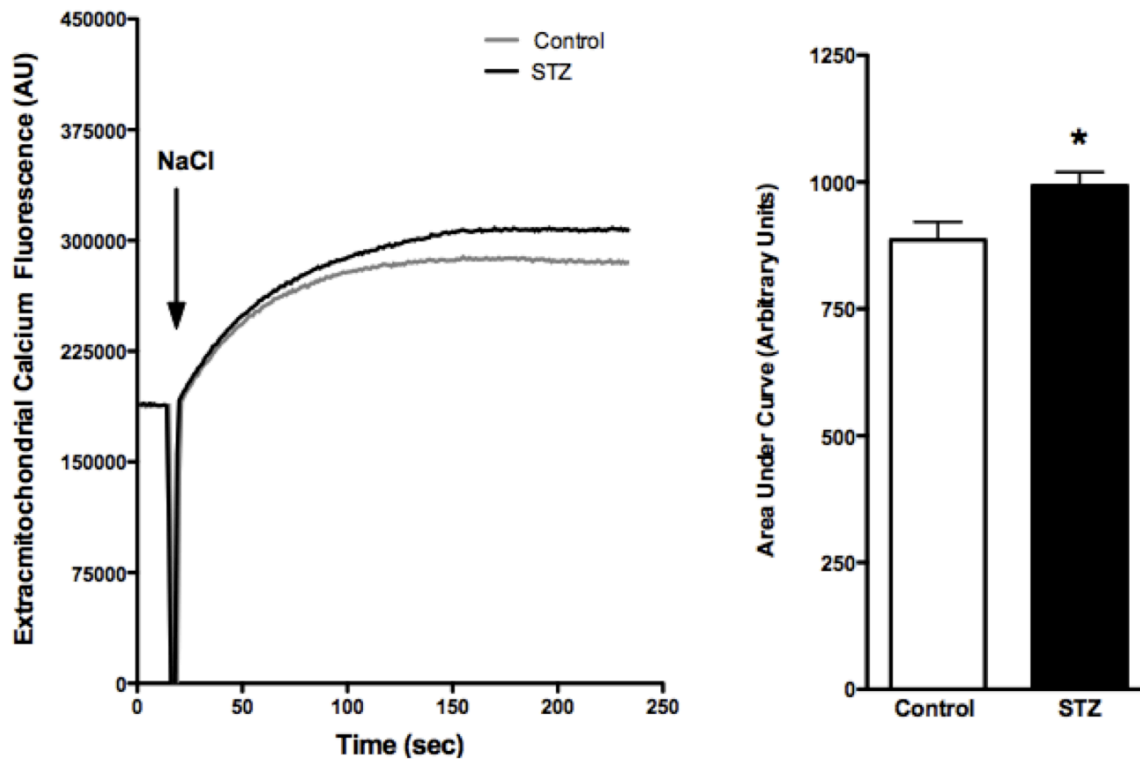
Figure 4.1 Representative infarct pictures and quantification of infarct sizes. TTC stained viable tissue bright red, while infarcted tissue appears pale in color. Lower dose KX (left); Higher dose KX (right); IA, infarcted area; AAR, area at risk; KX, ketamine-xylazine; data are presented as mean $\pm$ SEM; *, $P<0.05$ versus lower dose; N=7 in each group.
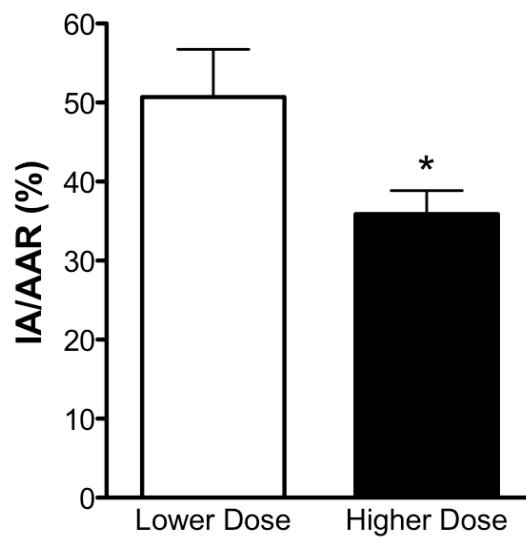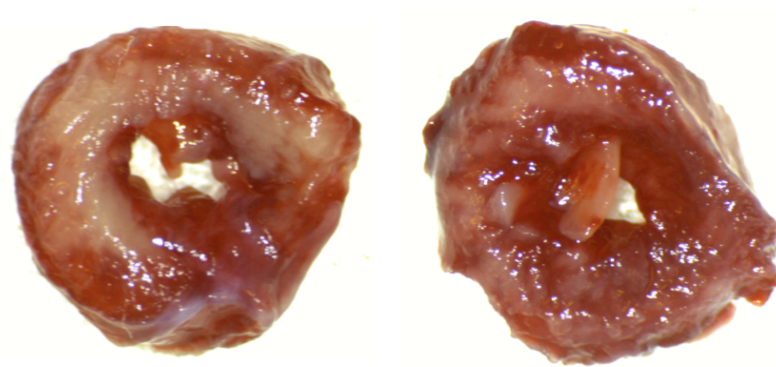
Table 2.1 Animal characteristics.   BW, body weight; HW, heart weight; FG, fasting glucose; C, control; STZ, diabetic; *, P<0.05 versus control

	C	STZ
BW (g)	348 $\pm$ 6.8	307 $\pm$ 12.4*
HW/BW (mg/g)	5.29 $\pm$ 0.12	4.44 $\pm$ 0.17*
FG (mg/dL)	114 $\pm$ 1.4	495 $\pm$ 21.9*

Table 2.2 Baseline and end of protocol hemodynamics. C, non-diabetic; STZ, diabetic; LVDP, left ventricular developed pressure; +dP/dt, maximal rate of left ventricular contraction; -dP/dt, maximal rate of left ventricular relaxation; CF, coronary flow. Data are presented as mean $\pm$ SEM; there were no significant differences between groups; N=5-10 in each group.

Baseline Hemodynamics

	C	STZ
LVDP (mmHg)	123+11.7	119+9.3
+dP/dt (mmHg/sec)	4312+444	3580+367
-dP/dt (mmHg/sec)	-2791+223	-2266+254
CF (mL/min/g)	8.3+0.7	8.8+1.5

Hemodynamics at the end of reperfusion

	C	C+N	C+B	C+M	STZ	STZ+N	STZ+B	STZ+M
LVDP (mmHg)	30 ± 7	22 ± 4	34 ± 3	36 ± 6	26 ± 3	25 ± 3	24 ± 4	32 ± 9
+dP/dt (mmHg/sec)	768 ± 96	734 ± 101	913 ± 216	1239 ± 195	816 ± 126	703 ± 117	789 ± 174	758 ± 173
-dP/dt (mmHg/sec)	-535 ± 63	-518 ± 73	-704 ± 63	-786 ± 101	-489 ± 63	-456 ± 57	-475 ± 75	-456 ± 57
CF (mL/min/g)	3.8 ± 0.4	2.5 ± 0.3	3.7 ± 0.2	4.1 ± 0.2	4.3 ± 0.7	3.7 ± 0.2	4.0 ± 0.6	4.3 ± 0.8

Table 4.1 Doses of ketamine/xylazine used in the lower and higher dose groups and hemodynamic parameters of the guinea pig heart taken at baseline and one hour into reperfusion. K, ketamine; X, xylazine; LVDP, left ventricular developed pressure (mmHg); +dP/dt, maximal rate of left ventricular contraction (mmHg/sec); -dP/dt, maximal rate of left ventricular relaxation (mmHg/sec); coronary flow (mL/min/g wet weight). Data are shown as mean $\pm$ SEM. *, $P<0.05$.

	Lower Dose K-85mg/kg X-15 mg/kg i.p.	Higher Dose K-200mg/kg X-60mg/kg i.p.	P-value
Baseline			
LVDP	82 $\pm$ 8	96 $\pm$ 10	0.28
+dP/dt	1947 $\pm$ 187	2138 $\pm$ 97	0.41
-dP/dt	-1512 $\pm$ 146	-1579 $\pm$ 85	0.71
Coronary Flow	6.3 $\pm$ 0.6	7.0 $\pm$ 0.6	0.43
Reperfusion			
LVDP	30 $\pm$ 8	49 $\pm$ 5	0.04*
+dP/dt	833 $\pm$ 186	1190 $\pm$ 94	0.13
-dP/dt	-576 $\pm$ 120	-876 $\pm$ 70	0.04*
Coronary Flow	4.5 $\pm$ 0.9	4.3 $\pm$ 0.6	0.86

▥ 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

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

RGC/jd

enclosure

Animal Care and Use Committee

212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

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

May 20, 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, "Cardiac Arrhythmias and Reactive Oxygen Species Production in Guinea Pig Myocardium", (AUP #Q269) was reviewed by this institution's Animal Care and Use Committee on 5/20/10. The following action was taken by the Committee:

"Approved as amended"

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