Mechanisms Underlying The Cardioprotective Effect of Inhibiting Soluble Epoxide Hydrolase

by

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ABSTRACT

Ischemic heart Disease (IHD) remains a major cause of illness and death worldwide. Therefore, therapeutic agents to protect against myocardial ischemia are needed. Arachidonic acid (AA) is metabolized by cytochrome P450 (CYP) epoxygenase into the biologically active epoxyeicosatrienoic acids (EETs). EETs are further metabolized by soluble epoxide hydrolase (sEH) into the less active dihydroxyeicosatrienoic acid (DHET). Literature shows that sEH suppression and/or EETs maintain a cardioprotective effect by enhancing cell survivability and inhibiting cell death. However, the exact mechanism is still unknown. In this thesis, we investigated the potential mechanisms underlying the cardioprotective effect mediated by sEH inhibition in young mice. The main focus was to investigate if sEH suppression maintains mitochondrial efficiency after myocardial ischemia. sEH suppression was induced either pharmacologically by using sEH inhibitor or genetically by targeted deletion. Left anterior descending coronary artery (LAD) ligation was used to induce myocardial ischemia. Our results demonstrated that both pharmacological and genetic suppression of sEH mediate cardioprotective events through maintenance of mitochondrial efficiency. We showed that sEH inhibition prevents systolic dysfunction following ischemic injury by preserving the mitochondrial pool in the non-infarct region of the heart. Furthermore, inhibiting sEH preserved insulin sensitivity in post-MI hearts reflecting enhanced cardiac metabolism thereby

suggesting there was activation of physiological recovery from ischemic insult.

DEDICATION

To my husband Peter E. Ghaly, as well as my whole family for their many years of support, encouragement and prayers.

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5,6-EET	5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid
8,9-EET	8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid
11,12-EET	11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid
14,15-EET	14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AEPU	1-adamantanyl-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)) urea
Akt	Protein Kinase B
AMPK	cAMP activated protein kinase
ATP	Adenosine triphosphate
AUDA	12-(3-adamantan- 1-yl-ureido)-dodecanoic acid
AUDA-BE	12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester
BAD	Bcl-2-associated death promoter
BAEC	Bovine aortic endothelial cells
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large

BID	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
Ca^{2+}	Calcium
CABG	Coronary artery bybass surgery
CcO	Cytochrome c oxidase
CoA	Coenzyme A
COX	Cyclooxygenases
cPLA2	Cytoplasmic phospholipase A2
CS	Citrate synthase
CVD	Cardiovascular diseases
CYP	Cytochrome P450
DHET	Dihydroxyeicosatrienoic acid
DMSO	Dimethyl sulphoxide
DNA	DeoxyriboNucleic acid
EET	Epoxyeicosatrienoic acid
EF	Ejection fraction
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
eNOS	Endothelial nitric oxide
EPHX	Epoxide hydrolase
EpOME	Epoxyoctadecanoic acid
ERK	Extracellular signal regulated kinases
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
Fe-S	Iron-Sulfur

FFA	Free fatty acids
FMN	Flavin mononucleotide
FS	Fractional shortening
G-6-P	Glucose-6-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK-3β	Glycogen Synthase Kinase-3β
H^-	Hydride ion
H^+	Proton
HB-EGF	Heparin-binding EGF-like growth factor
HETE	Hydroxyeicosatrienoic acids
HR	Hypoxia-reoxygenation
HSLAS	University of Alberta Health Sciences Laboratory Animal Services
IHD	Ischemic heart disease
IPC	Ischemic preconditioning
IPO	Ischemic Post Conditioning
IR	Ischemia reperfusion
IRES	Internal ribosomal entry site
K_{ATP}	ATP-sensitive potassium channel
KO	Knock-out
LA	Linoleic acid
LAD	Left anterior descending coronary artery
LOX	Lipoxygenases
LV	Left ventricle
LVDP	Left ventricular developed pressure
LVEDD	Left ventricular end-diastolic diameter

LVEDV	Left ventricular end-diastolic volume
LVESD	Left ventricular end-systolic diameter
LVESV	Left ventricular end-systolic volume
MAPK	Mitogen-activated protein kinases
mEH	Microsomal epoxide hydrolase
MI	Myocardial Ischemic
mitoK$_{ATP}$	Mitochondrial ATP-sensitive potassium channel
MMP	Metalloproteinase
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mtRNA	Mitochondrial RNA
Na^+	Sodium
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OsO4	Osmium tetroxide
P/O	Phosphorous to Oxygen
PGs	Prostaglandins
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C
PKG	Protein Kinase G
pmKATP	plasma-membrane ATP-sensitive K^+ channels
pro-HB-EGF	pro-Heparin-binding EGF-like growth factor
RCP	Respiratory control ratio
RISK	Reperfusion Injury Salvage Kinase

ROS	Reactive oxygen species
SAFE	Survival activating factor enhancement
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
sEH	Soluble epoxide hydrolase
sEHi	sEH inhibitor
SEM	Standard error of mean
Src	Sarcolemma
STAT-3	Signal transducer and activator of transcription-3
TAC	Thoracic aortic ligation
*t*AUCB	Trans-4-(4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid
TCA	Tricarboxylic Acid Cycle
TNF-α	Tumour necrosis factor alpha
TPAU	1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea
TTC	2, 3, 5-Triphenyltetrazolium chloride
TX	Thromboxanes
UA-8	13-(3-propylureido)tridec-8-enoic acid
VCAM-1	Vascular cell adhesion molecule
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
WHO	World Health organization
WT	Wild Type
XIAP	X-linked inhibitor of apoptosis
ΔΨm	Mitochondrial membrane potential

CHAPTER 1

INTRODUCTION

1.1 General Background

Cardiovascular disease (CVD) represents a major cause of morbidity and mortality worldwide (1-2). According to the Heart and Stroke Foundation of Canada, CVDs represent 29% of all deaths in Canada. This means that every seven minutes someone dies from heart diseases. The World Health organization (WHO) reported that CVDs are the main cause of death worldwide representing about 31% of all global deaths. CVDs have large impact on an individual's life, as well pose an economic burden to society. Therefore, more research is required to understand the pathophysiology of these diseases, which may potentially lead to the development of new pharmacotherapies.

CVDs are defined as disorders that affect both the heart and blood vessels. About 54% of CVDs deaths are due to ischemic heart disease (IHD). IHD results from injury caused by a decreased blood flow to the heart due to an obstruction from blockages such as atherosclerotic plaques or stenosis in the coronary arteries. Following an acute myocardial ischemic (MI) event, fast reperfusion to re-establish blood flow to the heart is critical. Pharmacological agents like thrombolytics that destroy the clot or percutaneous coronary intervention (i.e. balloon angioplasty) that open up the occluded region are commonly used (3). Interestingly, reperfusion itself can cause significant cardiomyocyte injury and even cell death (1). Calcium channel blockers, β-blockers, ACE inhibitors and other treatments are used to decrease the workload by the heart and its oxygen demand. Despite over 40 years of

research, there has been little success in translating therapeutic agents from the lab to the clinic (5). Novel strategies continue to be explored including investigation into signaling pathways regulating cell survival that may reduce the effects of IR injury.

Mitochondria are important organelles that play a crucial role in human physiological processes. Damage following IR injury results in mitochondrial dysfunction that may lead to cell death (2). Mitochondria are the main site for energy metabolism in the heart, providing cardiomyocytes with the fuel needed to sustain contractile activity (6). Mitochondria regulate energy supply and demand in the heart maintaining cardiac contractility and ionic homeostasis (7). There is growing interest in studying the changes in mitochondrial function in response to different diseases (i.e myocardial ischemia). Understanding the mitochondrial physiology and pathophysiology may give rise to new mitochondrial-targeted therapies (6).

The focus of this thesis is to study novel mechanisms underlying the cardioprotective effect of arachidonic acid (AA) metabolites known as epoxyeicosatrienoic acids (EETs). Furthermore, it provides experimental data demonstrating that inhibiting soluble epoxide hydrolase (sEH), an enzyme that degrades EETs, results in maintaining mitochondrial efficiency and function following ischemic injury.

1.2 EPOXYEICOSATRIENOIC ACIDS

1.2.1 Metabolic Formation of EETs

Arachidonic acid is a 20-carbon polyunsaturated fatty acid that is incorporated in the plasma membrane of cells (8-9). Upon stimuli, like ischemia, AA is released and activated by the action of phospholipase A2 (10). Cyclooxygenases (COX) and lipoxygenases (LOX) are well characterized enzymes involved in AA metabolism (11). COX metabolizes AA to the prostaglandins (PGs), prostacyclin and thromboxane (TX) (12). LOX metabolizes AA into leukotrienes, lipoxins and hydroxyeicosatrienoic acids (HETE) (13). A third pathway of AA metabolism involves the cytochrome P450 (CYP) monoxygenase system, which metabolizes AA into EETs and HETEs (14-17). (Figure 1.1)

CYP enzymes are a superfamily of proteins that metabolize a range of exogenous as well as endogenous compounds (18). CYP were first recognized as hepatic enzymes responsible for metabolizing various exogenous compounds including drugs and carcinogens (19-20). CYPs are important for the metabolism of various endogenous compounds such as steroids, fatty acids and hormones (21). CYP isozymes are expressed in the heart, brain, kidney and lung with known biological function (22-23). CYP2J2, CYP4A11, CYP2E1, CYP1A1 and CYP2C8, CYP2B6 and CYP2C9 mRNAs are expressed in the cardiovascular system (CVS) (24). CYP2C and CYP2J

families are the main families responsible for AA epoxidation into EETs in the CVS (14, 25-26).

EETs arise from the olefin epoxidation of AA where one molecule of oxygen is added to one of the four double bonds. There are four regioisomers of EETs (5,6-; 8,9-; 11,12-; and 14,15-EET) occurring either as R,S or S,R enantiomers (27) The CYP2C subfamily has a significant role during epoxidation reactions in mammals. The CYP2C8 isozyme is capable of generating 11,12- and 14,15- EETs regioisomers in equal ratios in humans (27). CYP2J subfamily and specifically CYP2J2, produce 14,15-EETs in 76% compared to 11,12- EETs (28). Among approximately 57 different CYP genes identified in the human genome, CYP2J2 has received much attention for its role in AA metabolism in the cardiovascular system (29). CYP2J2 is found to be highly expressed in the human heart and human polymorphism studies have shown an association between CYP2J2 polymorphism and cardiovascular diseases indicating their importance for the CVS (30).

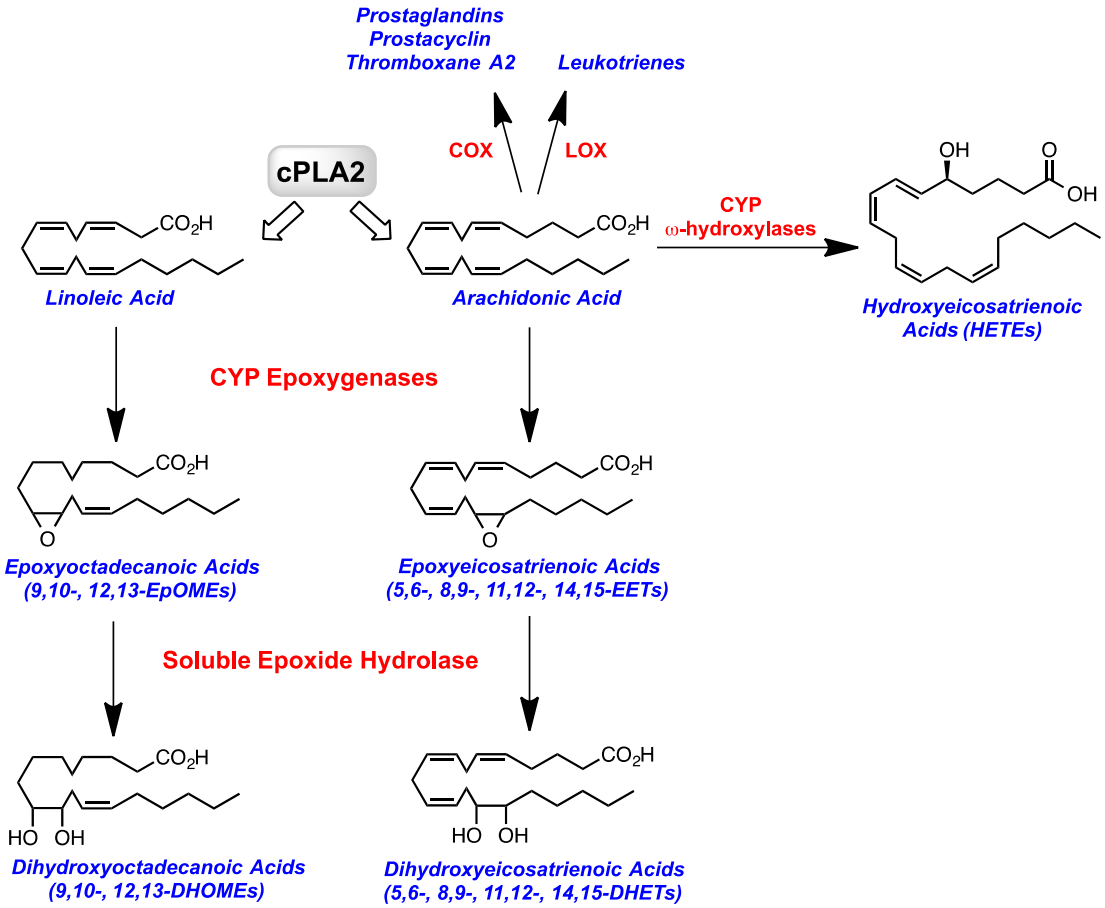

Figure 1.1: Catalytic activity of CYP epoxygenase and sEH: Activation of cytosolic phospholipase A2 (cPLA2) in cardiomyocytes leads to the release of membrane-bound fatty acids into the cytosol and their subsequent metabolism by CYP epoxygenases to form biologically active eicosanoids. These epoxyeicosanoids are further hydrolyzed by soluble epoxide hydrolase into the less biologically active dihydroxyeicosatrienoic.

1.2.2 Soluble Epoxide Hydrolase (sEH)

Epoxide hydrolases are a family of enzymes responsible for catalyzing the hydrolysis of epoxides into their dihydrodiol metabolites (31). Epoxide hydrolases are found in most organisms and are highly expressed in multiple tissues and organs. Studies have revealed the presence of at least five different epoxide hydrolases, however, the two of that have received the most attention for their physiological and pathological functions. These are the microsomal epoxide hydrolase (mEH) and the soluble epoxide hydrolase (sEH) (31).

mEH is encoded by the *EPHX1* gene on the long arm of chromosome 1 (32). It belongs to the α/β hydrolase fold enzymes with a molecular weight of about 51 kDa (33). It consists of 455 amino acid residues (34) with its catalytic site at the C-terminal (35) and the N-terminal acting as a membrane anchor (34). The main function of mEH is hydrolyzing xenobiotic epoxides as drugs, toxicants and procarcinogenic contaminants. The known functions of mEH primarily involve inactivation of endogenous and exogenous epoxides that can damage DNA and proteins (36). Moreover, previous studies demonstrate that mEH has some activity towards epoxy fatty acids such as EETs (37-38).

sEH is encoded by the *EPHX2* gene which is located on chromosome 8p21-p12 (39). sEH also belongs to the α/β hydrolase fold enzymes and it has a molecular weight of about 62 kDa, which consists of 554 amino acids

and makes a homodimer (40). sEH has 2 catalytic sites, the C-terminal has epoxide hydrolase activity and the N-terminal shows a phosphatase activity, however, its substrates remain unknown (40-41). (Figure 1.2) mEH and sEH have overlapping epoxide substrates, but sEH tends to be more active in the biotransformation of endogenously active epoxides into their inactive dihydroxydiols (42). Epoxides of low molecular weight fatty acids represent an excellent substrate to sEH, such as EETs the epoxides of arachidonic acid and epoxyoctadecanoic acids (EpOMEs) which are epoxides of linoleic acid (LA) (41, 43). The fact that sEH metabolizes biologically active fatty acids epoxides underscores its endogenous role, which is further supported by evidence demonstrating that sEH-mediated detoxification of xenobiotics is minimal when compared to mEH (44). (Figure 1.1)

sEH deactivates EETs by rapidly metabolizing them into the less active dihydroxyeicosatrienoic acids (DHETs) metabolites (27). EETs may undergo further removal by CYP-dependent metabolism, incorporation to plasma membrane, β-oxidation, binding to plasma and tissue proteins and chain elongation (27, 45). However, sEH is the predominant pathway of EET hydrolysis into DHET, which have reduced biological activity (46). It has been well documented that the suppression of sEH enzyme increases the levels of endogenous EETs by attenuating their degradation (47-48). Studies have showed that genetic deletion of sEH gene or direct pharmacological inhibition of sEH activity produced robust cardioprotective effects (49-52). Knocking out the *EPHX2* gene to generate sEH null mice showed direct evidence for the

role of sEH in treating hypertrophy and heart failure (50). Thus supporting the idea that sEH inhibitors (sEHi) are a potential pharmacological target for treating CVD.

Development of novel sEHi has resulted in numerous compounds; which include several urea, amide and carbamate based structures with demonstrated sEH inhibitory activity (53). The first generation sEHi contained chalcone oxides and glycidols, which were potent compounds but were rapidly inactivated by glutathione and glutathione transferases (54). X-ray crystallography revealed within the structure of the sEH enzyme that urea could be used as a central pharmacophore (53). Based on this knowledge, 12-(3-adamantan- 1-yl-ureido)-dodecanoic acid (AUDA) was synthesized to target sEH. However, AUDA was limited during *in vivo* studies due to its poor metabolic stability, high melting point and low solubility in both water and organic solvents (55-56). The development of 1-adamantanyl-3-(5-(2-(2-ethoxyethoxy) ethoxy) pentyl)) urea (AEPU) showed better *in vivo* efficacy due to better solubility and lower melting point (56). However, development of Trans-4-(4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid (*t*AUCB) and 1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea (TPAU) resulted in enhanced oral bioavailability, improved metabolic stability and potency with nanomolar activity (IC50= 1.3± 0.05 nM) (57). Potency was assessed by measuring EET/DHET levels in plama. Six hours after oral adminstration, 1 $mg.kg^{-1}$ *t*AUCB showed similar potency to 10 $mg.kg^{-1}$ of AUDA (48). This indicates that *t*AUCB is at least 10 times more potent than AUDA. Evidence

demonstrated *t*AUCB was stable in blood with a long elimination half life ($t_{1/2}$ > 1400 minutes) after oral administration in drinking water (48). Recently, it was found that sEH dimerization is essential for its activity which may lead to the development of novel sEHi that are based on disrupting sEH dimerization (58).

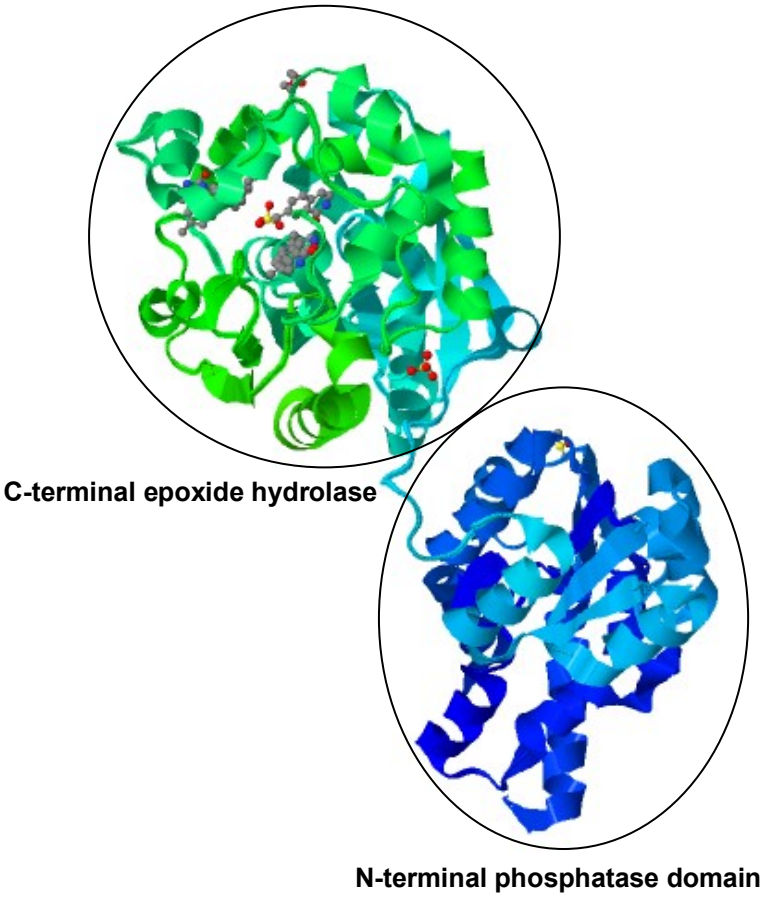

Figure 1.2: X-Ray Crystal structure of sEH: sEH consists of two domains; the C-terminal epoxide hydrolase domain and the N-terminal phosphatase domain (4).

1.3 CARDIAC ENERGY METABOLISM

1.3.1 Mitochondria

Mitochondria play a crucial role in human physiological processes. They are the main site for metabolism and energy production providing the cells with the fuel needed to survive (6). Mitochondria regulate energy supply and demand in the heart maintaining cardiac contractility and ionic homeostasis (7, 59). There is growing interest in studying the changes in mitochondrial function in response to injury and disease, which may give rise to new mitochondrial-targeted therapies (6, 60).

Structurally mitochondria consist of a phospholipid/protein bilayer, the outer membrane is smooth and the inner membrane is convoluted into cristea (59). About 75% of the inner membrane weight is composed of proteins involved in the electron transport chain (ETC) and ATP synthase. The cristae structure allows for an increased surface area providing high levels of mitochondrial respiration and energy production. The ETC consist of a series of enzymes and protein complexes that allow the transfer of electrons between donors and acceptors. (Figure 1.3)

Complex I (nicotinamide adenine dinucleotide (NADH) dehydrogenase ubiquinone oxidoreductase) is a multi-subunit protein that contains at least 45 subunits (61). The core of the enzyme is made of seven subunits that are mitochondrially-encoded, however, the remaining subunits are nuclear-encoded (62-63). It contains redox centers, as binuclear Fe-S centers,

tetranuclear Fe-S centers and flavin mononucleotide (FMN) molecules as prosthetic groups (61, 64). Complex I catalyzes the first step of ETC by coupling energy derived from the transfer of two electrons from NADH to ubiquinone-10 and translocating protons from matrix to intermembrane space (65). Complex I is one of the major sources of superoxide anion, thought to be the result of the iron- sulfur centers and the production of partially reduced semiquinone during ETC. Di-oxygen can react with flavin semiquinone producing superoxide or the Fe-S cluster can release electrons inadvertently (66).

Complex II (succinate: ubiquinone oxidoreductase; succinate dehydrogenase, SDH) has a unique structure and function compared to other respiratory complexes, which are encoded by nuclear DNA (67). Complex II has no direct role in the generation of proton motive force but is part of another energy converting pathway called the TCA, citric acid cycle or Krebs cycle (68). Complex II functions as a link between the ETC and the TCA cycle, by reducing ubiquinone to ubiquinol and oxidizing succinate to fumarate in TCA producing two electrons. This fuels the downstream complexes III and IV in ETC. Under aerobic conditions, electrons are carried by the FAD and FE-S clusters onto membrane bound ubiquinone (68-69). SDH consists of four subunits and redox centers such as the covalently bound flavin adenine nucleotide (FAD), Fe-S clusters and flavoprotien sulfhydryls (68). The two large subunits, flavoprotein subunit (SDH-A) and Fe-S protein subunit (SDH-B) are hydrophilic and contain the catalytic function of

the SDH enzyme (70). The other two subunits are anchor proteins that play an important role in the interaction between complex II and quinines (71).

Complex III (Cytochrome bc1; ubiquinol cytochrome c oxidoreductase, cyt bc1) consists of eleven subunits, three of them have redox centers which are subunit I (cytochrome b with two B-type hemes), subunit IV (cytochrome c1 with a C-type heme), and subunit V (the Rieske protein containing a [2Fe-2S] iron-sulfur cluster (72). It is considered a central enzyme in the ETC as it catalyzes the transfer of electrons from ubiquinol to cyt c along with translocation of protons across the inner mitochondrial membrane (65, 72). Another important facet of complex III is that it is bound to cardiolipin, which is necessary for the maintenance of cyt bc1 structure and its function and activity (73).

Complex IV (Cytochrome c oxidase (CcO) is a protein-phospholipid complex consisting of 13 subunits. Three key subunits (I, II and III) which are large and mitochondrially encoded. These form the redox active centers (Cu_A, cyt.a, cyt.a_3 and Cu_B) in the central core of the complex and are surrounded with ten smaller subunits that are nuclear-encoded (74). Complex IV catalyzes oxygen reduction in which electrons are transferred from redox centers to O_2, however, several intermediates are formed including the peroxyl and ferryl forms. Coupled with oxygen reduction, there is uptake of eight protons from the mitochondrial matrix. Four protons are translocated to the intermembrane space and the other four are used to reduce 2 oxygen molecules to water (65, 75).

NADH and $FADH_2$, generated from glucose and fatty acid oxidation, are the main source of high energy electrons for the ETC. Electrons in the form of hydride ions (H^-) pass through membrane bound electron carriers in a series of redox reactions until they reach oxygen which acts as final electron acceptor. Electrons from NADH first pass through complex I which in turn shuttles them through coenzyme Q into complex III. Electrons from $FADH_2$ are generated from complex II and pass to complex III as well. Electrons are then shuttled again through cytochrome c to complex IV (76). These redox reactions release energy creating an electrochemical proton gradient by pumping protons across the inner membrane. The resulting proton motive force drives ATP synthase to phosphorylate ADP to ATP by pumping the protons back into the mitochondrial matrix (77). Oxygen acts as a final acceptor for the energy depleted electrons (in the form of hydrogen) forming water. (Figure 1.3) During ischemic stress, several uncoupling proteins can lead to proton leak, changes in mitochondrial electrochemical gradiant and membrane potential (76). This can lead to dephosphorylation of ATP and ROS generation which results in energy wasting and more destruction to the mitochondria.

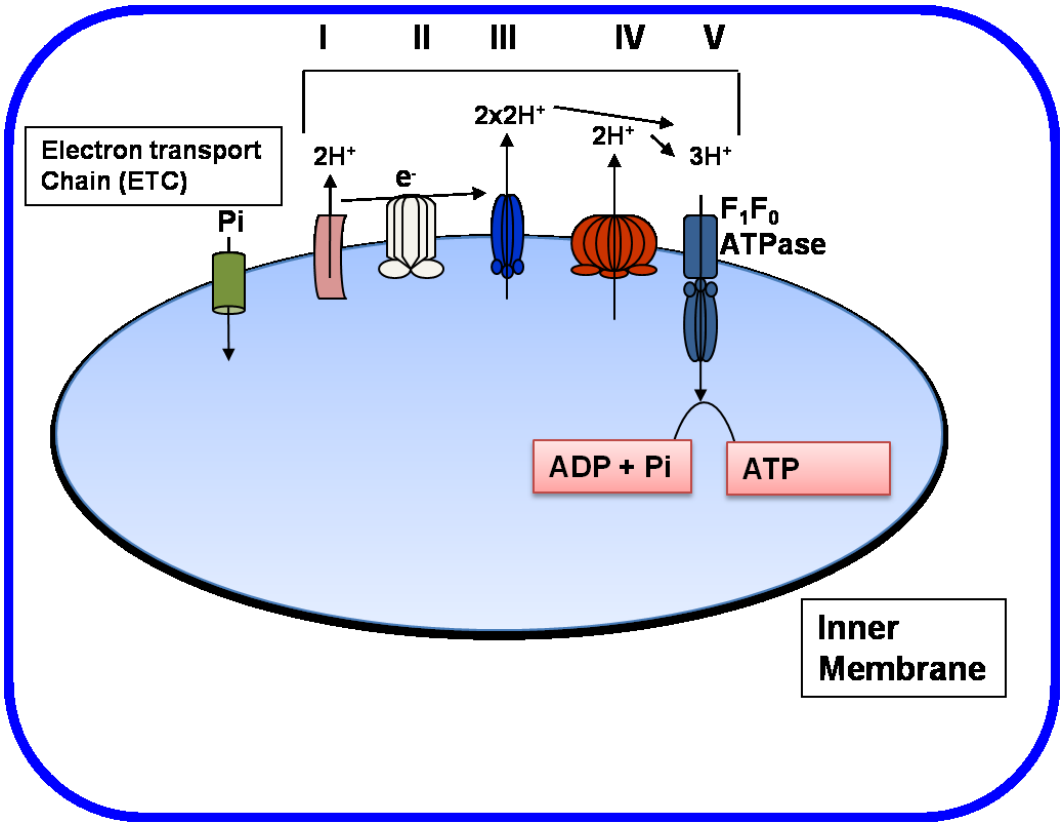

Figure 1.3: Electron transport chain: Electrons produced from glucose and fatty acid oxidation are shuttled through the electron transport chain creating a proton motive force. Three protons are required to pass through the ATP synthase in order for Pi and ADP to form an ATP molecule.

1.3.2 Substrate Metabolism And Cardiac Efficiency

The heart requires high energy to sustain uninterrupted contraction, basal metabolism and ionic homeostasis. Hence, not surprisingly, mitochondria occupy about 30% of the cardiomyocyte (78) compared to skeletal muscle mitochondria which represent about 2% of the cell volume. The cardiac muscle metabolizes carbon substrates; such as carbohydrates, fatty acids, amino acids or ketone bodies to meet its high energy demand (79). Indeed, the myocardial energetic requirements are regulated by cardiac fatty acid and glucose metabolism. Fatty acids and carbohydrates act as energy substrates that are metabolized by various dehydrogenase reactions during fatty acid β-oxidation and glucose oxidation respectively. Oxidation of fatty acids and carbohydrates produce reducing equivalents (protons and electrons) are transferred to the mitochondria via flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH). The balance between fatty acid β-oxidation and glucose oxidation is crucial for optimal myocardial function (79-81).

In fatty acid β-oxidation, the free fatty acid moiety in the cytosol is activated by esterification to CoA through ATP dependent processes by a family of fatty acyl-CoA synthase enzymes producing fatty acyl CoA (79). This fatty acyl CoA is then carried to the mitochondria through a complex of proteins using carnitine as shuttle mechanism (82). In the mitochondrial matrix, fatty acyl CoA undergoes fatty acid β-oxidation by a sequence of mitochondrial enzymes that sequentially shortens fatty acyl CoA molecules by

2 carbon units producing acetyl-CoA molecules and reducing equivalents (NADH and $FADH_2$) (79).

Glucose metabolism is initiated by glucose entry to the cytosol where it is phosphorylated via hexokinase producing glucose -6-phosphate (G-6-P). G-6-P is then catabolized by glycolysis via a series of enzymes producing pyruvate or lactate under aerobic or anaerobic conditions respectively. The metabolism of one glucose molecule through glycolysis produces 2 moles of ATP. Glucose oxidation is initiated when pyruvate produced in glycolysis under aerobic conditions is transported via a monocarboxylate carrier to the mitochondrial matrix where it can undergo oxidative decarboxylation by the pyruvate dehydrogenase complex producing acetyl CoA (80, 83-84).

Acetyl CoA produced from fatty acid β-oxidation and glucose oxidation is further metabolized by the TCA cycle producing more reducing equivalents and ATP. Reducing equivalents act as electron donors for electron transport chain and oxidative phosphorylation producing ATP. Hydrolysis of ATP, produced from mitochondrial oxidative phosphorylation, provides the cardiac muscle with more than 95% of its required energy. So, the ability of the cardiac cells to produce ATP and convert it into mechanical work reflects the cardiac efficiency (80).

Cardiac efficiency is represented by the P/O ratio (phosphorous to oxygen ratio) which is defined as the number of molecules of ATP produced through oxidative phosphorylation per the reduction of one atom of oxygen through the ETC. This value is influenced by the nature of the energy

substrate (fatty acid versus carbohydrates) that supply the electron transport with reducing equivalents (85). For instance, the oxidation of one palmitate molecule through β-oxidation produce 105 molecules of ATP, however, the oxidation of one glucose molecule through glucose oxidation produces 31 molecules of ATP. In spite of that the P/O ratio of palmitate is less than that of oxygen revealing that palmitate is less efficient substrate for ATP production as its oxidation requires much greater oxygen utilization than that of carbohydrates (80, 86).

From this, it is evident that the balance between metabolism mechanisms and the levels of carbohydrates and fatty acid oxidation is a crucial regulator of cardiac efficiency.

1.3.3 Glucose and Fatty Acid Oxidation

The heart is capable of rapidly switching between different substrates and mechanisms of energy production according to substrate availability and hormonal changes. In the fed state, the body produces insulin which stimulates glucose oxidation. However, in the fasted state, the heart primarily relies on fatty acid oxidation which supplies the heart with approximately 70% of its energy requirements (87). When fasting is prolonged the heart utilizes lactate and ketone bodies more prominently. Perturbations in this metabolic flexibility are believed to alter cardiac efficiency, and have been shown to be associated with many cardiac diseases such as IHD, heart failure and diabetic cardiomyopathy.

Ischemic stress stimulates the release of catecholamines elevating the level of plasma norepinephrine. Catecholamines increase free fatty acids by decreasing the release of insulin and increasing plasma levels of hydrocortisone (88). These alterations lead to adipose tissue lipolysis generating high level of plasma free fatty acids increasing their delivery to the myocardium (80, 89). The increase in FFA alters the balance between glucose oxidation and β-oxidation in favor of β-oxidation. As FFA is considered a less oxygen-efficient substrate (86), it is believed that increased FFA and β-oxidation exaggerate ischemic injury (90). Moreover, hypoxic cardiomyocytes consume more ATP when fatty acids levels are elevated to protect the heart from the accumulation of lipids (91).

When ischemia is prolonged, the oxygen deficiency limits both β-oxidation and glucose oxidation, making anaerobic glycolysis the predominant pathway for ATP production. Glycolysis generates a decreased amount of ATP in absence of oxygen to maintain cardiac contraction and ionic homeostasis. However, in prolonged or severe ischemia, the hydrolysis of the glycolytically-derived ATP uncoupled from subsequent pyruvate oxidation leads to generation and accumulation of lactate and protons causing intracellular acidosis (92). The decreased ATP production limits the function of various ATPase enzymes leading eventually to altered ionic homeostasis and calcium overload. Also, cardiac myofilament responsiveness to Ca^{2+} decreases due to intracellular acidosis. These lead to loss of cardiac

contractility during ischemia and may decrease the ability of the myocardium to recover during reperfusion (80, 93).

1.4 INVOLVEMENT OF MITOCHONDRIA IN CARDIOPROTECTION

1.4.1 Cardioprotection

Cardioprotection can be defined as "*all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage*" (94). In 1970s, E Brunwald talked about ways to limit injury from IR saying *"From the clinical point of view, is the finding that the severity and extent of myocardial ischemic injury resulting from coronary occlusion could be radically altered not only by pretreatment of the animal but also by an appropriate intervention as late as 3 hr after the coronary occlusion" (95).* Later on, the term conditioning was introduced including, pre-, post- and remote conditioning, as well as, pharmacological conditioning.

Murray et al introduced the concept of ischemic preconditioning (IPC) (96). In an attempt to induce more injury to their model of IR, they discovered short alternating episodes of ischemia and reperfusion prior to a long period of ischemia and reperfusion produced a cardioprotective effect. The data showed IPC results in a reduction in infarct size when compared to the control group (96). Subsequent studies revealed that IPC also reduced arrhythmias and maintained left ventricular function post-MI (97). When applying IPC to

patients undergoing coronary artery bybass surgery (CABG), it was found that IPC improves cardiac function after the surgery and decreases the need for inotropes. However, the fact that IPC needs to be applied just before the onset of ischemia, which is hard to predict, limits its clinical use to specific surgeries. Nevertheless, IPC is widely considered the most effective and reproducible method for limiting IR injury until now. IPC was effective in a variety of species, including rats (98), mice (99), pigs (100), sheep (101) and humans (102).

Ischemic post conditioning (IPO) was described in 2003 by Zhao et al due to the need for clinically applicable techniques to protect the heart from IR injury (103). IPO is applied by induction of sublethal episodes of ischemia and reperfusion early during reperfusion. As I discussed before that although reperfusion is essential however it causes oxidative stress, calcium accumulation and apoptosis (103). However, IPO was shown to confer a cardioprotective effect against IR injury by attenuating the adverse mediators of reperfusion injury (103). Clinically, IPO results in preventing reperfusion arrhythmias (104).

In spite of the effectiveness of IPC and IPO, they not always preferred as they are invasive. Direct conditioning to the heart is involved in IPC and IPO. Interestingly, studies revealed the cardioprotective effect of remote ischemic conditioning (RIC) which includes applying brief episodes of ischemia in an organ or tissue remote from the heart (105). This can be applied by inflating blood pressure cuff on the arm or leg to 200 mmHg for

alternating cycles of ischemia for 5 minutes followed by 5 minutes of reperfusion (105). Furthermore, pharmacological agents as adenosine, opioids, ACEi, bradykinin, erythropoietin, volatile anesthetics and PKCε agonists are cardioprotective against IR injury (106). However, none of them are clinically used for the treatment of MI due to limited clinical evidence (106).

There is large body of evidence demonstrating that conditioning (pre-, post-, remote-) triggers endogenous signaling mechanisms that activate cardioprotective effects. Numerous proteins and signaling pathways are involved in conditioning-mediated cardioprotection, some important proteins are PI3K, Akt, glycogen synthase kinase-3β, ERK1/2, mitogen activated protein kinase (MAPK), protein kinase C (PKC), protein kinase G (PKG), endothelial nitric oxide (eNOS), janus kinase and signal transducer and activator of transcription (JAK-STAT) (106-107).

Two major signaling pathways that mediate their effect by mitochondrial stabilization are the reperfusion injury salvage kinase (RISK) pathway and the survival activating factor enhancement (SAFE) pathway (108). The RISK pathway activated by both IPC and IPO, involves key kinases like Akt and ERK1/2 that inactivates GSK3β and mitochondrial permeability transition pore (mPTP) through a number of agents that promote cardiomyocytes survival (109-110). The SAFE pathway associated with IPC targets mitochondria by inhibiting mPTP. SAFE is activated by tumour necrosis factor alpha resulting in activation of signal Janus kinase (JAK) and

transducer and activator of transcription-3 (STAT-3) (110). STAT-3 activation upregulates transcription of cardioprotective proteins (111).

Mitochondria are responsible for cell survival and death; as such it is not surprising they have a critical role and the vast majority of studies investigating cardioprotection representing an important effector (107). Because mitochondria play an important role during conditioning-mediated cardioprotection, the development of novel therapeutic agents that target the mitochondria function is being pursued.

1.4.2 The Pathophysiology of Myocardial Ischemia

Myocardial ischemia is a condition encompassed by reduced blood supply to the heart muscle limiting oxygen and nutrient delivery as well as preventing the efficient removal of metabolic cellular waste. It is primarly caused by stenosis or occlusion of coronary arteries, usually due to deposition of atherosclerotic plaques. Coronary artery occlusion followed by early cardiac remodeling can progress to myocardial infarction and heart failure. Cardiac remodeling includes changes in the geometry and structure of the ventricles associated with ventricular dilation, wall stress, hypertrophy and ventricular dysfunction (112). Thus, cardiac remodeling is a major cause of damage to the heart following myocardial ischemia.

Several molecular and biochemical reactions are triggered after coronary artery occlusion including ATP depletion, increased cellular acidity,

increased ROS generation, failure of ionic pumps and cellular calcium overload. These events disrupt ionic homeostasis and trigger cell death, where necrosis is the predominant cell, but not the only, death pathway found in non-reperfused MI (112). In a murine heart, the healing process is initiated 1-3 days after the ischemic insult triggering an acute inflammatory response involving infiltration of monocytes and neutrophils (113-114). Following the early inflammatory response, is the establishment of chronic inflammation (113), which is accompanied by fibroblast proliferation and deposition of extracellular matrix to initiate tissue repair which results in scarring in 2-3 weeks (112, 114).

Reimer and Jennings demonstrated that after coronary occlusion in a canine model, necrosis is initiated within 40 minutes and spreads within 3 hours as a wavefront from the endocardium to the pericardium (115). The expansion of the necrotic region results in stretching, thinning and dilation of the infarct causing infarct expansion. In a clinical trial, two-dimensional echocardiographic imaging on patients within 72 hours of the onset of the myocardial infarction symptoms, demonstrated that Infarct expansion was detected early after ischemia (116). They also showed that infarct expansion is a main cause of LV dilation and dysfunction post-MI (116). If the infarct expansion is severe, it may be fatal due to left ventricular free-wall cardiac rupture (117). In murine hearts, cardiac rupture can occur as early as 3 days post-MI (114).

Cardiac remodeling may occur in both the infracted and non-infarcted regions of the heart (112). One of the major consequences of cardiac remodeling and LV dysfunction is the increased risk of progressive remodeling in post-MI patients. The affect accentuates infarct expansion characterized by wall thinning and LV dilation (112). Furthermore, this will lead to more fatal complications including heart rupture, lethal arrhythmias and cardiogenic shock, eventually causing heart failure and death (118).

1.4.3 Mitochondrial Physiology And Pathophysiology in IHD

An inadequate blood supply to the heart causes an imbalance between oxygen supply and oxygen demand. Reduced or no blood flow to the heart results in reduced ATP production and disturbances in ionic homeostasis. The effects of IHD result in an energetically compromised heart with an imbalance between its demand and production. Current therapy focuses on reperfusion by thrombolytic drugs or surgery which can cause further mitochondrial damage causing cellular death and cardiac dysfunction (119-120), together with treatments that reduce workload and oxygen consumption by the heart muscle, such as β-blockers, calcium channel blockers and angiotensin converting enzyme (ACE) inhibitors. Other treatments increase blood supply to the heart, such as nitroglycerin and calcium channel blockers. These treatments reduce the imbalance between oxygen supply and demand which results in improvement in cardiac function and prevention of reoccurrence of MI. Cholesterol lowering agents and anti-platelets are also used to prevent reoccurrence of atherosclerosis. However, no drug is used to

directly prevent cell death following ischemic injury. Interest in discovering novel therapeutic agents that target cardiac mitochondria with the intention of maintaining efficiency and inhibiting cell death has grown in recent years (121).

During ischemia, the lack of oxygen stimulates anaerobic glycolysis causing an increase in lactate production, altering the physiological pH and increasing the influx of hydrogen ions into the mitochondria. The acidic environment causes alteration in mitochondrial membrane potential and activation of the Na^+/H^+ exchanger to remove excess H^+. Subsequent, sodium accumulation in the mitochondria switch Na^+/Ca^{2+} exchanger to reverse mode, which are unable to extrude calcium leading to calcium overload (122). Calcium overload causes mitochondrial damage by activating phospholipases and proteases, which break down cellular and mitochondrial components.

The mPTP is a multiprotein complex whose exact structure is not clear. mPTP spans both mitochondrial membrane and includes adenine nucleotide translocator (ANT) and cyclophillin-D in its structure (123-124). The duration of mPTP opening determines different pathophysiology. mPTP opens briefly and rapidly under normal physiological conditions. During ischemic stress, transient mPTP opening results in loss of small (<1500 Da) molecules (123-124). However, during IR, mPTP opening is prolonged which results in loss of mitochondrial membrane potential and release of pro-apoptotic factors. This can explain the association between mPTP opening and cell death (124).

Opening of mPTP leads to mitochondrial swelling and subsequent rupture leading to cell apoptosis or necrosis (125).

During ischemia, the mPTP in the inner mitochondrial membrane remains closed due to the inhibition effect of the high levels H^+ on the opening of mPTP (126). However, during reperfusion there is a state of calcium overload, oxidative stress and the pH returns to normal leading to uncoupling of electron flow, opening of the mitochondrial permeability transition pore (mPTP). This results in an influx of osmolytes leading to swelling of the inner membrane and rupture of the outer membrane (126-128), which eventually cause cell necrosis due to the release of pro-death proteins in the cytosol triggering inflammation of the tissue (119-120, 127, 129). Oxidative stress and DNA damage during IR activate proapoptotic proteins, Bax and/or Bak, which permeabilize the mitochondrial outer membrane (121, 130-131). Upon activation, they undergo conformational changes and translocate to the mitochondria. Bax can be oligomerized and interact with other Bax molecules in the mitochondrial membrane leading to its cleavage by calpain which results in permeabilization of the outer membrane (132). Other Bcl-2 proapoptotic protiens such as Bid and Bim interact with Bax-type proteins at the outer mitochondrial membrane causing a conformational change in the multidomain proapoptotic members. This will lead to intermembranous oligomerization and permeabilization of the outer mitochondrial membrane (133). Moreover Bax and Bid can interact with the mitochondrial lipid bilayer, especially cardiolipin, through irreversible covalent bonds (134). These

mitochondrial outer membrane pores increase its permealization allowing the release of cytochrome c and other proapoptotic proteins leading to cell death.

Mitochondrial DNA damage is another complication of ischemia and IR injury that results in impaired mitochondrial function. Mitochondria have their own genomic system. The mitochondrial DNA (mtDNA) consists of a closed-circular double-stranded DNA molecule (about 16.5 kb) that contains two promoters (135-136). The light strand promoter and the heavy strand promoters are essential for transcription of mtRNAs encoding 13 subunits of oxidative phosphorylation (135). The light strand promoter also allows the transcription of RNA primer that is necessary for the replication of mitochondrial DNA. Mitochondrial DNA (mtDNA) damage leads to decline in mitochondrial RNA (mtRNA) transcripts and protein synthesis, which alters the mitochondrial function and causes its injury and death (135-136). Oxidative stress and excessive production of ROS during IR injury can cause mutations to the mitochondrial DNA (136). ROS-induced mtDNA mutations interfere with normal DNA replication. Mitochondrial DNA is more susceptible than nuclear DNA to oxidative stress due to the close proximity between the site of ROS production (electron transport chain) and the mtDNA. Another reason is poor repair mechanisms in the mtDNA rather than nuclear DNA and the lack of histone-like proteins (135). Histone-like proteins are chromatin complex organizations that act as barriers against ROS production (135, 137). mtDNA mutations may prevent replication or expression decreasing mtRNA and proteins important to ETC respiratory complexes. Inhibition of

mtDNA synthesis leads to mitochondrial dysfunction, resulting in further ROS generation and progressive cellular destruction (136-137).

In an ischemic mouse heart model, ETC enzyme activities were downregulated four weeks after LAD ligation (136). It was hypothesized to be due to downregulation of mtRNA caused as a consequence of DNA damage (136-138). A study using male-sprague rat hearts exposed to 30 minutes of global ischemia using Langendorff perfusion method, demonstrated a 20-30% downregulation of mtRNA during ischemia compared to control (138). During the transition from ischemia to reperfusion, there is a rapid burst in the ROS generation (139). This causes a biphasic process in protein biosynthesis and enzymatic activity of the mitochondrial electron transport chain (138). When rat hearts were exposed to IR using Langendorff perfusion, upregulation of mitochondrial ETC biosynthesis and enzymatic activity was observed. However, there was poor recovery of respiratory control index and recovery of contractility. This was suggested to be due to impairment of protein-protein interaction of the ETC caused by respiratory complexes I, II and III injury during reperfusion (138). This can be caused by oxidative stress that can induce some genes in internal ribosomal entry site (IRES) at the 5' untranslated region of mRNA to escape translational control and undergo rapid protein translation (140). In addition to the post-ischemic ROS-induced impairment of the 20S/26S-proteosomal activities that decrease its ability to degrade proteins which may result in accumulation of ETC proteins. Taken together, decreased mitochondrial function and efficiency is a main outcome

of ischemic injury. Mitochondrial dysfunction plays an important role in IHD and mitochondria represent an excellent target for protecting the heart against ischemic injury.

1.5 BIOLOGICAL EFFECTS OF EETs

1.5.1 EETs and Ischemia Reperfusion Injury

As previously discussed, hypoxia-reoxygenation (HR) or ischemia-reperfusion (IR) results in myocardial dysfunction. In vitro, ex vivo and in vivo studies suggested that EETs protect the heart and cardiomyocytes from IR and HR-induced injury.

In 1996, Wu et al first introduced that EETs in CYP2J3 overexpressed rats improve post-ischemic recovery and protect the heart from IR (141). Then the field opened up when Seubert et al showed a significant improvement in post-ischemic recovery of contractile function using Langendorff isolated heart model (142-143). Mice with cardiomyocyte-specific CYP2J2 overexpression have improved postischemic left ventricular recovery after 20 minutes of global ischemia followed by 40 minutes of reperfusion (143). Similar results were seen using sEH null mice and UA-8-treated mice in which left ventricular developed pressure (LVDP) was improved after IR (142, 144). It was suggested that sEH suppression confer its cardioprotective effect through inhibiting the degradation of EETs and that EETs activate PI3K signaling pathway as well as sarcolemmal K_{ATP} channels (142). They suggested that EET mediated its cardioprotective effect through activation of

mitoK_{ATP} and p42/p44 MAPK signaling pathways (143). Administration of 11,12- and 14,15-EETs activates K_{ATP} channels which results in a significant decrease in myocardial infarct size in dogs (145) and rats (146) exposed to ischemia via temporary coronary artery occlusion (145-146). Further in vivo studies showed that sEHi can provide cardioprotection against IR in rats through several pathways targeting the mPTP (147).

In vitro, direct administration of 11,12-EET, sEHi (dicyclohexylurea) or overexpression of CYP2J2 in cultured bovine aortic endothelial cells exposed to HR showed significant attenuation of HR-induced oxidative stress and apoptosis (148). Similar results were obtained by direct administration of 14, 15-EETs in HL-1 cells and neonatal cardiomyocytes, which suggested that EETs mediate their antiapoptotic effect through the PI3K/Akt pathway (149). 11-12 EETs showed protection against HR-induced apoptosis in ex vivo culture from myocardium of human, rat and mouse. This study confirmed EETs confer antiapoptotic effects through activation of the PI3K/Akt pathway and ATP-sensitive potassium channels (K_{ATP}) (150)

1.5.2 EETs and Cell death

Further in depth mechanistic studies have demonstrated that EETs mediate their effects through pathways targeting the mitochondria. Previous studies on H9c2 cells using sEH inhibitors demonstrate EETs maintain mitochondrial membrane potential ($\Delta\Psi_m$) and limit mitochondrial dysfunction following cellular stress (151). Moreover, administration of sEH inhibitor (BI00611953) reduces cellular stress and death markers such as ROS

generation, proteasome activity, and cell death (151). Using cardiomyocyte-specific CYP2J2 overexpressing mice, it was suggested that EETs can inhibit mitochondrial damage by maintaining mitochondrial dynamics limiting mitochondrial swelling and fragmentation following IR injury (119).

It was suggested that the activation of PI3K/Akt pathway plays an important role in the EET-mediated antiapoptotic effect. Studies on BAEC cells showed that EETs activate the MAPK and PI3K/Akt pathways following cellular stress (152). Activation of MAPK and PI3K/Akt pathways inhibits glycogen synthase kinase-3beta (GSK-3β) which targets the mPTP complex limiting its opening and mediates a cardioprotective effect (142, 153). EETs can also enhance Ca^{2+}-sensitive K^+ channels (BK_{Ca}) channels activities after HR injury which increase K^+ uptake and decrease Ca^{2+} overload (142). It is well known that Ca^{2+} stimulates the translocation of the proapoptotic protein BAD from the cytosol to the mitochondria by dephosphorylating it which leads to opening of the mPTP complex. As well as, dephosphorylated BAD forms a heterodimer with the antiapoptotic Bcl-2 and Bcl-x_L inactivating them allowing Bax/Bak-triggered apoptosis. Thus, limiting Ca^{2+} overload can have a potential cardioprotective effect by limiting the opening of mPTP and preventing mitochondrial localization of BAD and subsequent caspase -3 activation and apoptosis (151, 154-155).

Activation of the PI3K-Akt pathway can exert its antiapoptotic function by inhibiting translocation of Bax to mitochondria. Bax translocation to the mitochondria induces cytochrome c release either by forming a pore through

oligomerization or by opening VDAC channel (156). Recent studies showed that activated Bax induces cytochrome c release by forming proteolipid pores in the outer mitochondrial membrane leading to mitochondrial outer membrane permeabilization (157). EET-mediated activation of the PI3K/Akt pathway can have a cardioprotective effect through inhibition of cytochrome c release which limits caspase activation and apoptosis (156). Other mechanisms of EET-mediated antiapoptotic effects include activation of X-linked inhibitor of apoptosis (XIAP). XIAP is an important antiapoptotic protein which acts by blocking caspases 9 and 3 (149). A study by Dhanasekaran et al showed that the HL-1 cells and neonatal cardiomyocytes treated with EETs are protected against apoptosis (149). They demonstrated the involvement of PI3K, Akt, BAD, XIAP, and caspase-9 in attenuating HR-induced cell death and apoptosis (149).

Recently our group has shown another mechanism of EET-mediated protection through activation of autophagy. Autophagy represents a biologically beneficial strategy to preserve cellular integrity during stress. Starved cells treated with EETs showed an enhanced predominance of autophagic response and reduced apoptotic response, thus shifting the cellular death pathway to favor cell survival. The signaling pathway involved the activation of pmK_{ATP} channels and AMPK (158).

Besides an antiapoptotic effect, EETs have been shown to stimulate mitogeneic effects and cell proliferation in renal epithelial cells involving activation of Src kinase (159). EETs were shown to induce mitogenesis in

cultured rat glomular mesangial cells through stimulation of Na^+/H^+ exchange (160).

1.6 THESIS OVERVIEW

1.6.1 Rationale

Cardiovascular diseases represent a major cause of illness and death in Canada, they result in approximately 70,000 deaths per year (161-163). The vast majority of the related mortalities (54%) are attributed to adverse effects associated with ischemic injury (161-163). Key organelles, like mitochondria, are a major contributing factor to the pathogenesis of IHD (164). As mitochondria are critical to cell survival and heart function, obtaining a clear understanding about MI induced dysfunction is important for developing new strategies to improve outcomes and prevent further cardiac damage. In the heart, mitochondria provide the primary source of energy that fuels the contractile apparatus and act as key regulators of cell survival and death (165-166). Therefore, maintaining mitochondrial integrity is vital for cellular homeostasis and cardiac performance. Ischemic injury can cause significant mitochondrial damage resulting in cellular death and cardiac dysfunction,

Arachidonic acid (AA) is a polyunsaturated fatty acid normally found esterified to cell membranes that can be released in response to several stimuli including ischemia (167). Free AA can then be metabolized by cytochrome P450 epoxygenases to epoxyeicosatrienoic acids (EETs),

eicosanoids, which are metabolized to dihydroxyeicosatrienoic acids (DHETs), via soluble epoxide hydrolase (sEH), or incorporated into membranes (56). Accumulating evidence indicates EETs have important functional roles in maintaining cardiac homeostasis and preventing ischemic injury. Our findings indicate the importance of EETs in limiting mitochondrial damage caused by IR injury and provide insight into new mechanisms that regulate mitochondria. To date our lab have established that: (1) EETs minimize damage to mitochondrial ultrastructure caused by IR injury (119), (2) EETs limit the loss of mitochondrial membrane potential ($\Delta\Psi m$) and opening of the mPTP caused by cellular stress (119), (3) EETs minimize doxorubicin increased mitochondrial fragmentation, membrane depolarization and caspase-3 activation. Recent studies have confirmed the beneficial effects of sEH inhibitors against IR injury, hypertension, diabetes and stroke (168-171). Thus, an alteration in the production and/or elimination of EETs may improve steady-state cellular levels of these bioactive eicosanoids, potentially influencing cardiac function.

1.6.2 Hypothesis

We hypothesize that suppression of EET degradation, using genetic deletion or pharmacological inhibitors of sEH, initiate a cardioprotective response against ischemic injury; whereby sEH inactivation preserves mitochondrial function and efficiency, leading to a subsequent improvement in postischemic cardiac function.

1.6.3 Thesis Aim

1. To investigate if suppression of sEH either genetically or pharmacologically confers a cardioprotective effect against ischemic injury
2. To assess the role of sEH inhibition in maintaining mitochondrial function after ischemic injury
3. To investigate the role of sEH inhibition in maintaining the cardiac metabolic profile

CHAPTER 2

Inhibition of Soluble Epoxide Hydrolase Limits Mitochondrial Damage and Preserves Function Following Ischemic Injury

ABSTRACT

Purpose: Cardioprotective effects of epoxyeicosatrienoic acids (EETs) toward acute myocardial ischemic injury have been recognized; however, the precise mechanism(s) are still largely unknown. Our study investigates the protective effects of EETs by inhibiting soluble epoxide hydrolase (sEH), the enzyme responsible for EET metabolism, following surgical occlusion of left anterior descending artery (LAD) of the heart.

Methods: Age matched 2 month old sEH *null* (KO) and littermate wild-type (WT) mice were utilized in the study. C57Bl/6 WT mice were administered an sEH inhibitor, trans-4-[4- (3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*AUCB; 10mg/L) or vehicle in drinking water for 4 days prior and 7 days post surgery. Mice from all groups were subjected to surgical occlusion of LAD, and cardiac function was assessed by echocardiography prior to and 7 days post surgery. Mice were euthanized on day 7 and heart tissues were dissected into infarct, peri-infarct (area at risk) and non-infarct (healthy) regions to assess cellular and sub-cellular structure by electron microscopy (EM). Hearts were collected and mitochondrial respiratory enzymes in complexes I, II, III, IV and citrate synthase activities were assayed following MI injury and respiration was assessed using a Clark-type electrode. Isolated working heart was used to measure the rates of glucose and palmitate oxidation.

Results: Hearts from *t*AUCB treated and sEH *null* mice showed significantly improved ejection fraction ($p<0.05$) and fractional shortening ($p<0.05$) compared to WT counterparts. Echocardiogram revealed less cardiac remodeling in *t*AUCB treated and sEH *null* groups evident by reduced left ventricular internal diameter ($p<0.05$) during both systole and diastole. EM data showed preservation of mitochondrial ultrastructure in the non-infarct region of all tested groups. Inhibition of sEH resulted in significant improvement in mitochondrial respiration, ATP content and ETC enzymatic activities ($p<0.05$) in the non-infarct region. Furthermore, sEH suppression results in restoration of insulin sensitivity ($P<0.05$). These data suggest EETs have a cardioprotective effect by maintaining mitochondrial integrity and respiratory function.

Conclusion: Inhibition of sEH or genetic deletion of sEH gene provides cardioprotection against long-term ischemia, associated with preserved post-ischemic cardiac function and maintaining mitochondrial efficiency.

2.1 INTRODUCTION

Arachidonic acid (AA) is a polyunsaturated fatty acid found in the phospholipid domain of cell membranes. Activation of cytoplasmic phospholipase A2 (cPLA2) triggers the release of AA, which is further metabolized into a vast array of lipid mediators. The prostaglandin-endoperoxidase synthase/cyclooxygenase, the lipoxygenase and the cytochrome P450 (CYP) pathways are the predominant enzyme systems that metabolize AA into prostanoids, leukotrienes and eicosanoids (172). Epoxygenases such as CYP2J and CYP2C isozymes metabolize AA into four regioisomers of biologically active epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET) (173). Removal of EETs can occur by conjugation, chain elongation, β-oxidation, and esterification, resulting in reincorporation into phospholipid membranes. However, the predominant pathway of EET metabolism is the formation of less active vicinal diol compounds by epoxide hydrolases, most notably the soluble epoxide hydrolase (sEH) (173). There is a large body of evidence demonstrating that EETs have an important role acting as lipid mediators in the cardiovascular system. Considering EETs are rapidly metabolized, research to develop sEH inhibitors has been an approach to increase cellular EET levels (38, 174). Inhibition of sEH has been associated with decreasing atherosclerotic plaque lesions in mice aortae (175), decreasing blood pressure in hypertensive mice (176) and protecting against ischemic injury (170, 177-178). Other effects of inhibiting sEH include vasodilatory, pro-angiogenic and cell migratory effects

(54, 120, 179). However, the mechanism(s) and/or specific subcellular targets of these effects remain elusive.

In the heart, mitochondria provide the primary source of energy for fueling the contractile machinery. These dynamic organelles undergo continuous fusion and fission processes (dynamics), which are closely related to cellular energy demands and stress levels (166). As cardiomyocytes are terminally differentiated post-mitotic cells, maintenance of a healthy pool of mitochondria depends upon a delicate balance between newly generated organelles and efficient degradation of irreversibly damaged organelles (180). Mitochondria are strategic regulators of cell life and death given the fact that they play a central role in energy production, calcium homeostasis and stress adaptation (181). The heart's high-energy demand during normal function is met by a continuous supply of ATP mainly produced through oxidative phosphorylation in mitochondria (119-120). Of interest, mitochondrial dysfunction has been linked to numerous human diseases (182). During ischemic stress, several signaling pathways affect mitochondrial function and structure, which can impact ionic gradients and initiate cell death pathways (119-120). These changes lead to uncoupling of electron flow, opening of the mPTP and loss of cytochrome c, leading to mitochondrial dysfunction and eventually irreversible cell death (119-120, 129).

We have previously demonstrated that EETs enhance cardiomyocyte cell survival via a protective cascade targeting the mitochondria (152, 158, 179). Emerging evidence suggests the cardioprotective effect of EETs is due

to inhibition of mitochondrial damage. For instance, EETs limit mitochondrial damage and fragmentation following IR injury in CYP2J2 overexpressing mice compared to wild type littermates (119). In addition, EETs minimize doxorubicin-induced mitochondrial dysfunction and damage preventing cardiotoxicity (183). sEH inhibition has been shown to maintain mitochondrial membrane potential ($\Delta\Psi_m$) following cellular stress limiting mitochondrial dysfunction (151). The present study investigates the effect of sEH inhibition after myocardial ischemia demonstrating maintenance of mitochondrial efficiency.

2.2 MATERIALS AND METHODS

2.2.1 Animals

A colony of mice with targeted disruption of the *EPHX* gene (sEH *null*) are maintained at the University of Alberta (142). Commercially available C57BL/6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ). All studies were carried out using 2-3 month old mice weighing 25-30g. To pharmacologically inhibit sEH, 10 mg/L *t*AUCB (a kind gift from Dr. Bruce Hammock) was administered to WT mice in drinking water 4 days prior to surgery and continued for 7 days after surgery (57). Vehicle (DMSO; 1 ul/ml) was added to the drinking water of the sEH *null* and WT littermates. Experiments were conducted according to strict guidelines provided by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS).

2.2.2 Myocardial infarction (MI)

MI was induced by permanent occlusion of the proximal left anterior descending (LAD) coronary artery as described (184-185). Mice were anaesthetized, intubated and underwent left thoracotomy in which LV was exposed by opening the pericardium and the LAD was encircled and ligated. In sham-operated mice, LAD was encircled but not ligated. Animals were inspected at least 2 times per day and after 7 days were euthanized and hearts were sampled.

Hearts were collected from sham-operated and post-MI mice to isolate left ventricle and separate infarct, peri-infarct and non-infarct regions (186).

The different regions were separately flash-frozen using liquid nitrogen and stored at -80˚C for further analysis.

2.2.3 Infarct size analysis:

Hearts were sliced from apex to the point of ligation in 0.5-mm slices. Slices were then incubated in 1% triphenyltetrazolium chloride at 37°C for 10 minutes. In viable tissues, TTC is reduced by dehydrogenases to 1,2,5-triphenylformazan which has a brick red colour. In necrotic tissues, The TTC will remain white due to the absence of enzymes. Images were then analyzed using image J and infarct size was represented as a percentage of total LV (186).

2.2.4 Echocardiography Measurements

Non-invasive functional assessment was performed by transthoracic echocardiography using a Vevo 770 high-resolution imaging system with a 30 MHz transducer (RMV-707B; VisualsSonics). Isoflurane (0.8% by anaesthetic machine) was used to anaesthetize the mice during the recordings (186-187). To assess the change in cardiac function, echocardiography was carried 1 day prior to MI (baseline) and 7 days post MI. Left ventricular end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD) were obtained from M-mode images as well left atrial size was obtained by M-mode imaging in the parasternal long axis view.(187) Systolic function was assessed by calculating ejection fraction (%EF) and fractional shortening (%FS) using the following equations %EF = (LVEDV − LVESV/LVEDV) × 100 and %FS=

(LVEDD − LVESD/LVEDD) ×100 (188). Tei index was calculated as (isovolumic contraction time (IVCT) + isovolumic relaxation time (IVRT))/Ejection time (ET). Diastolic function was represented as early transmitral filling wave (E-wave) and late filling wave (A-wave) which were measured using pulsed-wave Doppler imaging as described in (188). VisualSonics software was used for the qualitative and quantitative measurements (186).

2.2.5 Mitochondrial Ultrastructure

Mitochondrial ultrastructure was assessed in several pieces from different regions of the left ventricle that were pre-fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, post-fixed in 2% osmium tetroxide (OsO_4) in 0.1M sodium cacodylate buffer, dehydrated in an ethyl alcohol series, embedded with epoxy resin and thermally polymerized as previously described (119). Ultra thin-sections (60 nm) were cut by a ultramicrotome (Leica UC7, Leica Microsystems Inc., ON, Canada) and then stained with 4% uranyl acetate and Reinold's lead citrate. The contrasted sections were imaged under a Hitachi H-7650 transmission electron microscope at 80 kV equipped with a 16 megapixel EMCCD camera (XR111, Advanced Microscopy Technique, MA, USA) was used for viewing the sections (119, 189).

2.2.6 Mitochondrial Function

Non-infarct regions were used for assaying mitochondrial enzymatic function (complexes I-IV and Citrate synthase) spectrophotometrically (190). Non-infarct regions of LV of Post-MI hearts and whole sham hearts were flash frozen in liquid nitrogen shortly after harvesting. Frozen tissues were crushed using mortar and pestle. Heart powders were then homogenized in ice-cold homogenization buffer (0.121 g of Tris, 0.15 g of KCl and 0.038 g of EGTA in 50 ml of distilled water, pH 7.4, 0.854 g of sucrose /10 ml of the buffer was added at the day of the experiment). Sample homogenates were centrifuged at 600 g for 10 min at 4ºC and supernatant was collected. Protein was then assayed using Bradford reagent.

Complex I activity was assayed by adding 40 ug of the sample supernatant proteins to 700 ul of distilled water in a 1-ml cuvette. 100 µl of potassium phosphate buffer (0.5 M, pH 7.5), 60 µl of fatty acid–free BSA (50 mg ml^{-1}), 30 µl of KCN (10 mM) and 10 µl of NADH (10 mM) were added. The reaction contents were mixed by inverting and baseline was recorded at 340 nm for 2 min. Then, 6 ul of ubiquinone (10 mM) was added to start the reaction and the decrease in absorbance was recorded for 5 min.

To assess complex II activity, 600 µl of distilled water, 50 µl of potassium phosphate buffer (0.5 M, pH 7.5), 20 µl of fatty acid–free BSA (50 mg ml^{-1}), 30 µl of KCN (10 mM), 50 µl of succinate (400 mM) and 10 ug of the sample supernatant proteins were added in a 1-ml cuvette. The whole mixture was incubated at 37°C for 10 min. Baseline activity was recorded at 600 nm

for 2 min. 4 µl of 12.5 mM DUB was added to start the reaction and the decrease in absorbance was recorded at 600 nm for 5 min.

Complex III activity was assessed by adding 730 µl of distilled water, 50 µl of potassium phosphate buffer (0.5 M, pH 7.5), 75 µl of oxidized cytochrome c, 50 µl of KCN (10 mM), 20 µl of EDTA (5mM, pH 7.5), 10 µl of Tween-20 (2.5% (vol/vol)) and 5 ug of the sample supernatant proteins in a 1-ml cuvette. Baseline was recorded at 550 for 2 min. 10 µl of 10 mM decylubiquinol was added to started the reaction and the increase in absorbance was recorded at 550 nm for 5 min

To assess complex IV activity 400 µl of distilled water, 500 ul of potassium phosphate buffer (100 mM, pH 7.0), 60 ul of 1 mM reduced cytochrome c. Baseline was recorded at 550 nm for 2 min. 2.5 ug of sample supernatant proteins were added to initiate the reaction and the decrease of absorbance was recorded immediately for 5 min at 550 nm

Citrate synthase activity was assayed by adding 300 µl of distilled water, 500 µl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (vol/vol)), 100 µl of DTNB, 30 µl of Ac CoA (10 mM) and 6 µg of the sample supernatant proteins in a 1 ml cuvette. Baseline activity was recorded at 412 nm for 2 min. The reaction was started by adding 50 µl of 10 mM oxaloacetic acid and the increase in absorbance was immediately recorded at 412 nm for 5 min.

Enzyme activities were calculated according to the following formula; enzyme activity (nmol min^{-1} mg^{-1}) = (Δ Absorbance/min × 1,000)/[(extinction

coefficient × volume of sample used in ml) × (sample protein concentration in mg ml^{-1})]. In which extinction coefficient of 6.2 mM^{-1} cm^{-1}, 19.1 mM^{-1} cm^{-1} , 18.5 mM^{-1} cm^{-1} , 18.5 mM^{-1} cm^{-1} and 13.6 mM^{-1} cm^{-1} were used for complex I, II, III, IV and Citrate synthase respectively.

ATP content was also assessed in non-infarct regions colorimetrically using ATP assay Kit (ab83355, Abcam Inc, Toronto, ON, Canada). Heart powders were homogenized in the provided buffer. Sample homogenate was centrifuged at 15000 x g for 2 min and the supernatent was processed for the ATP assay. Standard curve for ATP, as well as, reaction mixture were prepared according the kit manual in a 96-well plate. The plate was kept in the dark for 30 minutes and optical density (OD) was measured at 570 nm.

2.2.7 Mitochondrial isolation and respiration

Mitochondria were freshly isolated from hearts following sampling (31). Hearts tissues were harvested from anaesthetized mice then connective tissues and vessels were removed in ice-cold PBS. The hearts were minced in ice-cold isolation buffer (in mmol/l: sucrose 250; HEPES 10; EGTA 1, pH 7.4) added as 1 ml per heart. Fatty acid free BSA (5 mg/ml) was added to the isolation buffer shortly before starting the experiment. The minced hearts were gently homogenized in glass-glass homogenizer on ice to ensure isolation of undamaged mitochondria. In the next step, the hearts homogenates were centrifuged at 700xg for 10 minutes. The supernatents were further centrifuged at 10,000xg for 10 minutes. The resulting pellets were resuspended inthe isolation buffer and centrifuged at 10,000xg for 10

minutes again (191). The final pellets represent purified mitochondrial fractions, which were resuspended in 300 ul of respiration buffer (EGTA 0.5 mM, $MgCl_2.6H_2O$ 3 mM, taurine 20 mM, KH_2P0_4 10 mM, HEPES 20 mM, BSA 1 g.liter^{-1}, potassium-lactobionate 60 mM (120 ml of 0.5 M K-lactobionate stock solution liter^{-1}), mannitol 110 mM, dithiothreitol 0.3 mM, fatty acid-free BSA 1 mg ml^{-1}, pH 7.1) then sterilized by filteration, aliquoted and kept in -80ºC prior using. The mitochondrial samples were resuspended in the respiration buffer. The samples were kept at 4°C throughout the experiment. The protein concentration was then assayed using Bradford protein assay.

Mitochondrial oxygen consumption was measured in isolated mitochondria (50 ug of mitochondrial protein) added to a chamber connected to OXYGRAPH PLUS system controlled through PC (Hansatech Instruments Ltd, Norfolk, England). Respiration rates were measured at 30^0C in 2 ml of respiration buffer. Basal respiration was recorded after the addition of 5 mM malate and 10 mM glutamate as substrates for overall oxidative respiration. ADP-stimulated respiration was initiated by respiration addition of 0.5 mM ADP then recorded. The efficiency of coupled oxidative phosphorylation was calculated as the ratio between basal and ADP-stimulated respiration rates (192).

2.2.8 Immunoblot Analysis

Non-infarct regions of the LV were flash frozen using liquid nitrogen and crushed with a mortar and pestle on dry ice to be kept at -80˚C. The heart powder was then homogenized in ice-cold lysis buffer (158). After

homogenization, the homogenate was centrifuged at 10000 x g for 10 min. The protein concenentrantion in the supernatant was assayed using Bradford reagent. 20 ug of protein was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted (158). Cytosolic and mitochondrial fractions were prepared from frozen mouse hearts as described (158). Immunoblots were prepared using cytosolic (100 µg protein) or mitochondrial (25 µg protein) fractions and probed with antibodies to SDH-A, COX IV, CS (Abcam, Burlingame, CA, USA) and GAPDH (Cell signaling Technology, Inc., New England Biolabs, Ltd., Whitby, ON, Canada). Relative band intensities were assessed by densitometry using Image J (NIH, USA). Protein expression in vehicle treated controls were taken as 100% and compared with treated group.

2.2.9 Measurement of glucose oxidation and fatty acid oxidation

Hearts from both sham-operated and post-MI mice were isolated and perfused in Langendorff mode with krebs hanseliet buffer for approximately 20 minutes. During this time, the pulmonary vein was cannulated to connect the left atrium to a preload reservoir and switch the heart to the working mode. Isolated working hearts were perfused at a left atrial preload of 11.5 mmHg and an aortic afterload of 50 mmHg (34-35). The preload reservoir is wrapped in a heated jacket of 38.5ºC to keep the heart at approximately 36.7ºC.

The perfusate contained 5 mM [U-^{14}C] glucose, 1.2 mM [9, 10-^{3}H] palmitate, and 3% albumin. The palmitate was prebound to 3% fatty acid free

bovine serum albumin. First, hearts were subjected to aerobic perfusion without insulin for first 30 min, then 100 μU/ml insulin was added to investigate the response to insulin.

Glucose oxidation was determined by quantitative collection of the $^{14}CO_2$ released by the metabolism of [U-^{14}C] glucose. $^{14}CO_2$ was trapped using filter paper paper soaked in 1 M hyamine hydroxide. The hyamine hydroxide samples were counted using CytoScint scintillation cocktail (ICN). Palmitate oxidation was determined by measuring the amount of $^{3}H_2O$ produced from the metabolism of [9, 10-^{3}H] palmitate. $^{3}H_2O$ was separated from 9,10-[^{3}H] palmitate in the krebs hanseliet buffer KHB using separation into polar and nonpolar phases (193-194).

2.2.10 Statistical Analysis

Values expressed as mean ± standard error of mean (SEM). Statistical significance was determined by one-way ANOVA with Tukeys post hoc test was performed to assess differences between groups. Values were considered significant if $p < 0.05$.

2.3 RESULTS

2.3.1 sEH inhibition preserves cardiac function following myocardial ischemia

Baseline heart function in WT, *t*AUCB treated and sEH *null* mice was similar between all groups (Figure 2.1 and Table 1). However, WT mice had significantly worse cardiac function following myocardial ischemia compared to *t*AUCB treated and sEH *null* mice (Figure 2.1 and Table 1). At 7 days post-MI WT mice showed systolic dysfunction as determined by an increased LVESD (Figure 2.1A, 2.1B) and LV systolic volume (Table1), decreased EF (Figure 2.2A) and FS (Figure 2.2B). Inhibition of sEH either pharmacologically (*t*AUCB treated) or genetically (sEH null) attenuated the post-MI systolic dysfunction as shown by the significant preservation in EF (Figure 2.2A) and FS (Figure 2.2B). sEH *null* mice showed a significant increase in LVESD while *t*AUCB treated mice showed a trend of increase in LVESD although this was not statistically significant. (Figure 2.1B) Similarly, LV systolic volume (LV Vol;s) showed a significant preservation in the sEH *null* group but not in the *t*AUCB treated group (Table 1). WT mice demonstrated post-MI increases in LVEDD (Figure 2.1A, 2.1C), LV diastolic volume (Table 1), left atrial size (LA) (Table 1) and a decrease in mitral A velocity (Table 1) compared to baseline. Although not statistically significant, there was a trend of decreasing LVEDD upon sEH inhibition (Figure 2.1C). *t*AUCB treated groups showed a significant attenuation of post-MI alterations in LA size and mitral A velocity compared to the WT post-MI group (Table 1). We assessed the doppler-derived myocardial performance index (TEI index), defined as the sum of

isovolumic contraction time and isovolumic relaxation time divided by the ejection time index, and observed a marked increase in WT post-MI groups while inhibition of sEH prevented this effect (Figure 2.2C). The attenuation of cardiac dysfunction by sEH inhibition was not accompanied by any significant attenuation in infarct size as measured by TTC analysis (Figure 2.3A).

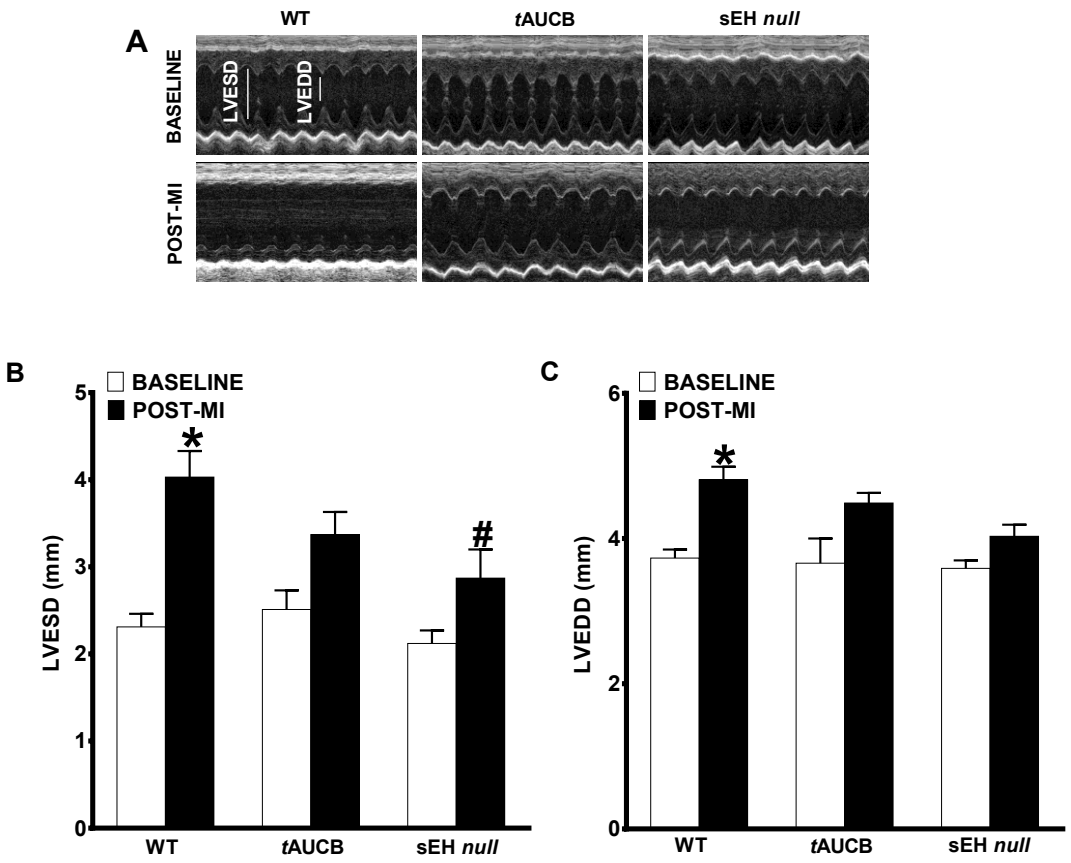

Figure 2.1: Effect of sEH suppression on preserving ventricular dimensions. Cardiac function of WT, *t*AUCB treated and sEH *null* mice were assessed one day before LAD ligation (Baseline) and seven days after LAD ligation (POST-MI). (A) Representative M-mode images revealed a decrease in left ventricular dilation and dysfunction upon sEH inhibition either pharmacologically (*t*AUCB) or genetically (sEH *null*) (B) Left ventricular end systolic dimension (LVESD) and (C) Left ventricular end diastolic dimensions (LVEDD) were improved upon sEH inhibition. Values are represented as mean ± S.E.M. N=6-9. Significance was $P<0.05$, *significantly different from its baseline, #significantly different from WT post-MI.

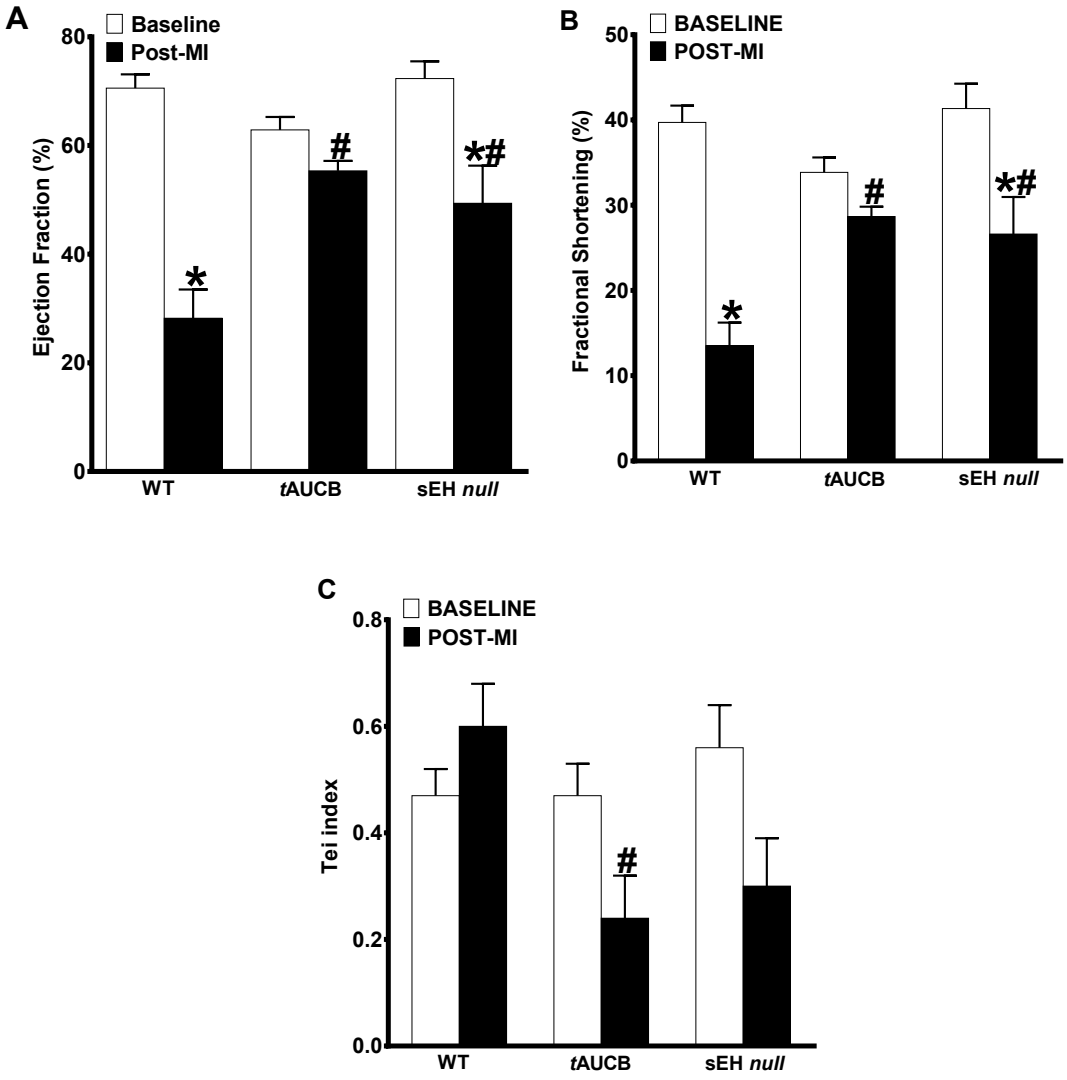

Figure 2.2: Effect of sEH suppression on cardiac function. Inhibition of sEH preserves contractile function of theheart post-MI. (A)Ejection Fraction (EF) and (B) Fractional shortening (FS) and (C) Tei index were improved upon sEH inhibition. Values are represented as mean ± S.E.M. N=6-9. Significance was $P<0.05$, *significantly different from its baseline, #significantly different from WT post-MI.

Table 1: Echocardiographic assessment of heart function in WT and sEH suppressed mice

	WT Baseline	***WT Post-MI***	***tAUCB Baseline***	***tAUCB Post-MI***	***sEH null Baseline***	***sEH null Post-MI***
Heart rate (beats/min)	*476.9 ± 17.4*	*430.3 ± 13.0*	*460.3 ± 19.0*	*426.8 ± 13.1*	*517.9 ± 19.8*	*493.1 ± 19.6*
LV Vol; d	*56.9±3.3*	*116.1±7.4**	*70.5±5.6*	*87.3±4.8#*	*54.7±4.0*	*72.2±7.6#*
LV Vol; s	*17.5 ±2.5*	*83.7 ±8.9**	*26.9 ± 3.2*	*39.2 ± 3.1*	*15.6±2.4*	*37.8±8.5#*
%EF	*70.6 ±2.6*	*28.2 ±5.3**	*62.9 ±2.4*	*55.4 ± 1.8#*	*72.3±3.2*	*49.4±6.9#*
%FS	*39.7 ± 2*	*13.5 ±2.7**	*33.9 ±1.8*	*28.7 ±1.2#*	*41.3±2.9*	*26.6±4.3#*
LV Mass (mg)	*72.9 ±3.6*	*77.4 ±10.2*	*83.3 ±6.3*	*80.7 ±7.2*	*69.3±3.9*	*86.0±9.3*
LA (mm)	*1.6 ±0.1*	*2.7 ±0.2**	*1.9 ±0.2*	*1.9 ±0.1#*	*2.1±0.1*	*2.0±0.2*
Mitral E Vel (mm/sec)	*727.7±35.4*	*638.6±51.2*	*744.3±30.3*	*628.8±47.2*	*811.5±25.4*	*677.7±26.5*
Mitral A Vel (mm/sec)	*445.3±26.5*	*216.5±53.1**	*444.8±39.8*	*480.7±58.9#*	*485.7±18.5*	*352.4±27.2*
IVRT (ms)	*14.5 ±1.8*	*18.5 ±1.5*	*14.3 ±2.2*	*15.6 ±1.1*	*13.4±0.5*	*14.7±1.04*
IVCT (ms)	*10.7 ± 1*	*12.9 ±2.2*	*8.6 ±1.2*	*8.6 ±1.0*	*7.1 ±0.7*	*4.9 ±0.8*
ET (ms)	*53.7 ±1.9*	*49.8 ±0.8*	*49.8 ±1.4*	*52.7 ±2.6*	*39.6 ±3.3*	*44.6 ±1.6*

*Values are represented as mean ± S.E.M. N=6-9. Significance was P<0.05, *significantly different from its baseline, #significantly different from WT post-MI.*

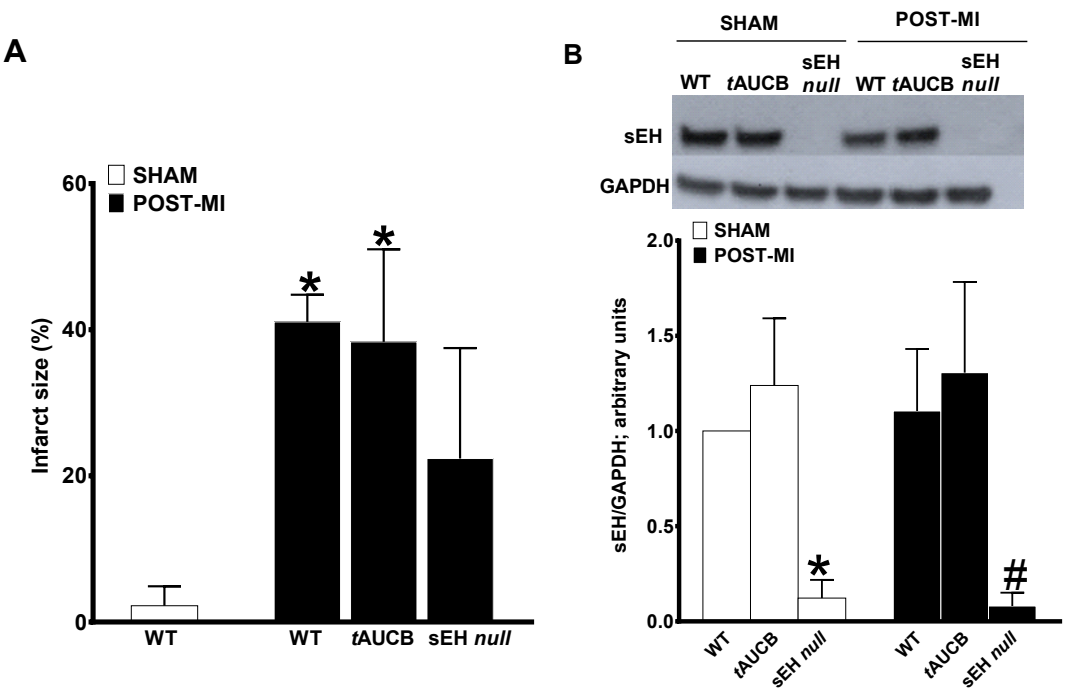

Figure 2.3: Infarct size and sEH expression in WT and sEH suppressed hearts (A) Quantification of infarct size was assessed from representative images of transverse heart sections stained with TTC. (B) Representative blots and the results of quantification showing successful deletion of sEH gene. Values are represented as mean ± S.E.M. N=3-4. Significance was $P<0.05$, *significantly different from sham WT, #significantly different from WT post-MI.

2.3.2 sEH inhibition protects the mitochondria from ischemic damage

To visualize the effect of ischemia on mitochondrial ultrastructure we assessed baseline and post-MI hearts using electron microscopy. Healthy and intact mitochondria were observed in the sham groups with no differences between WT, *t*AUCB or sEH *null* mice. Seven days post-MI, we dissected the left ventricle into infarct, peri-infarct and non-infarct regions. EM images demonstrate that the mitochondrial ultrastructure of the infarct region in the sEH *null* group was significantly more preserved than the infarct regions of the WT and *t*AUCB treated groups. In the peri-infarct region, the mitochondrial content was more preserved than the infarct region in all the groups, moreover, mitochondrial damage was more attenuated in *t*AUCB and sEH *null* mice than the WT group. The mitochondrial ultrastructure was not impacted in the non-infarct region of the 3 groups (Figure 2.4).

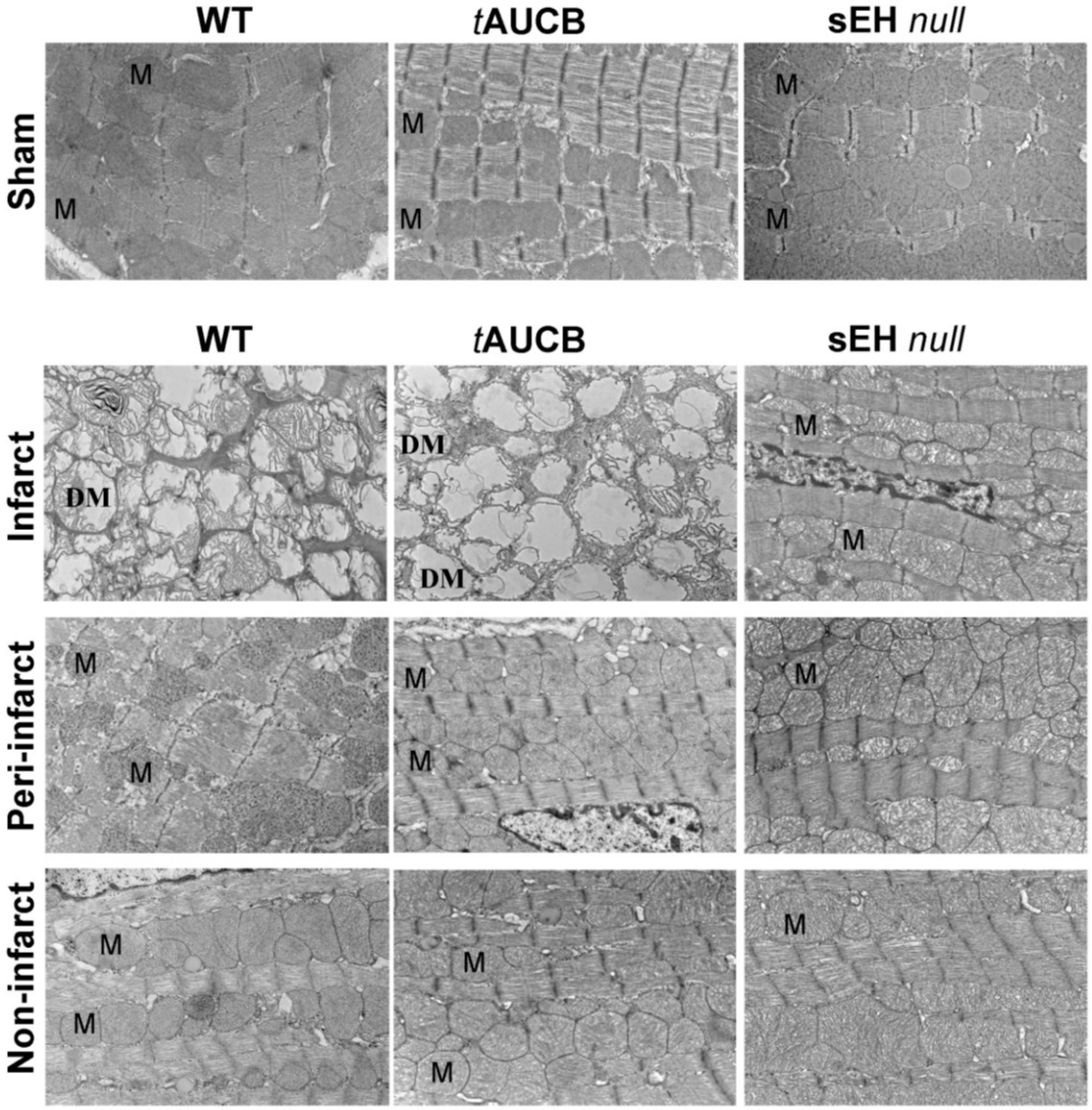

Figure 2.4: Effect of sEH suppression on mitochondrial integrity. Representative electron micrograph (EM) images of infarct, peri-infarct and non-infarct regions of the heart. (M) represents individual mitochondrion. (DM) represents damaged mitochondrion (Magnification=6000x).

2.3.3 sEH inhibition leads to preservation of mitochondrial efficiency in non-infarct region

In order to maintain cardiac contractility and function, the heart needs a healthy pool of mitochondria to supply it with the energy required for contraction. We first quantified the abundance of key mitochondrial proteins in the non-infarct region where mitochondrial ultrastructure was preserved. Consistent with EM images, there was no significant difference in the protein content between any of the groups in either sham or post-MI for citrate synthase, succinate dehydrogenase or cytochrome C oxidase expression (Figure 2.5). These observations suggest the pool of mitochondrial protein found within the non-infarcted region remains the same relative to controls.

Enzymatic activities of the mitochondrial respiratory chain were assessed in non-infarcted regions of the heart where no damage in the mitochondrial ultrastructure was observed. No significant differences were observed in the enzymatic activities between sham WT, *t*AUCB or sEH *null* groups (Figure 2.6A-E). However, there was a significant drop in citrate synthase (CS) activity in WT post-MI groups. This decrease was significantly attenuated in *t*AUCB treated and sEH *null* groups (Figure 2.6A). Similarly complexes I and II of the electron transport chain (ETC) showed a significant drop in their enzymatic functionality in WT post-MI groups, however, this was significantly attenuated in the *t*AUCB and sEH *null* mice (Figure 2.6B, 2.6C). Complexes III and IV were preserved from ischemic dysfunction in both WT and treated groups post-MI (Figure 2.6D, 2.6E).

Considering sEH inhibition attenuated the loss of catalytic activity of key enzymes involved in ATP production, we next measured respiration in isolated mitochondria. To ensure respiration rates were not attributed to low substrate availability, malate and glutamate were used to support basal respiration. WT post-MI groups showed a significant decrease in ADP-simulated respiration that was attenuated by inhibiting sEH with *t*AUCB or deletion in sEH *null* mice, which is reflected in the preserved respiratory control ratio (RCR). Enhancement in RCR was seen in the post-MI *t*AUCB and sEH *null* groups compared to WT mice (Figure 2.7A). Consistent with better mitochondrial respiration following inhibition or loss of sEH, ATP content in the non-infarct region of the LV was maintained in the *t*AUCB and the sEH *null* groups post-MI (Figure 2.7B).

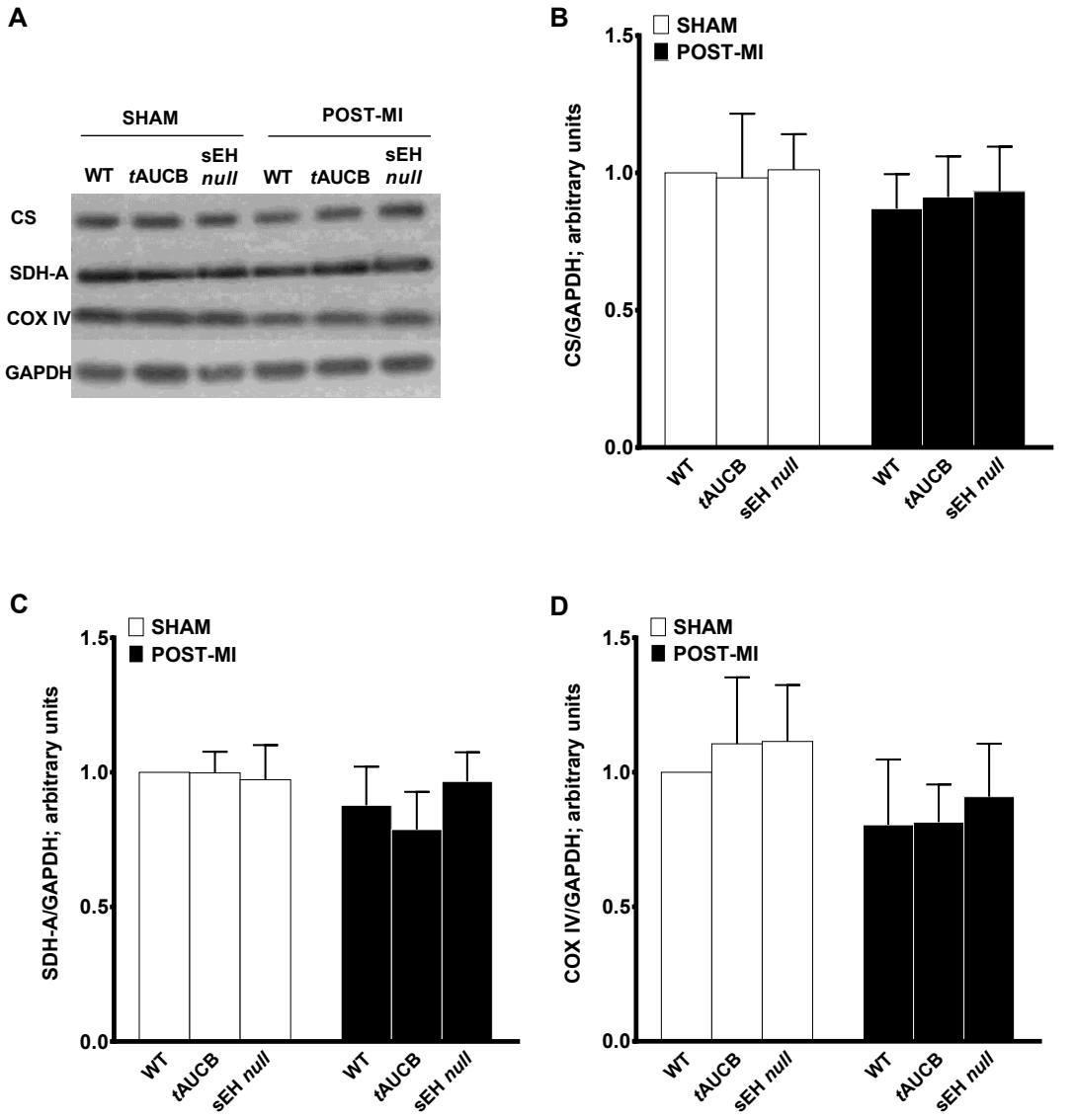

Figure 2.5: Mitochondrial protein expression in the non-infarct region of the left ventricle. (A)Representative western blots and the results of quantification showing (B) citrate synthase (CS), (C) succinate dehydogenase (SDH-A) and (D) COX-IV were observed in samples from non-infarct region of the heart seven days post-MI and samples from the Left ventricle of sham hearts, as detected by western blot. Values are represented as mean ± S.E.M. N=4.

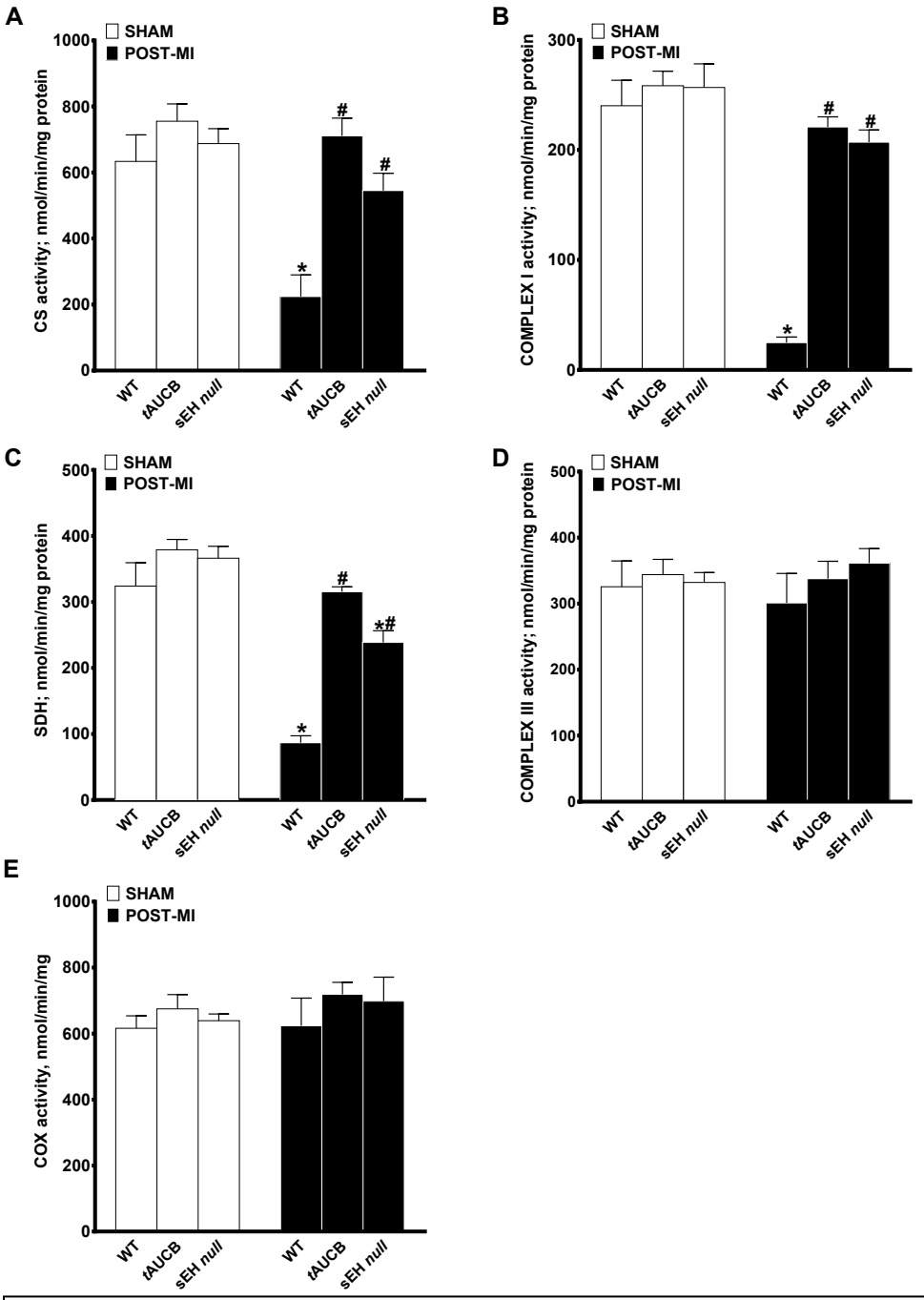

Figure 2.6: sEH suppression preserves a healthy pool of mitochondria during ischemic injury. Activities of key mitochondrial enzymes were assessed spectrophotometrically in the non-infarct region of the left ventricle of mice hearts 7 days after LAD ligation. Activities of (A) citrate synthase (CS), (B) complex I and (C) succinate dehydrogenase were improved in sEH inhibited-mice post-MI. (D) Complex III and (E) cytochrome C oxidase (COX) activities were not affected. Values are represented as mean ± S.E.M. N=4. Significance was $P<0.05$, *significantly different from respective control sham, #significantly different from WT post-MI

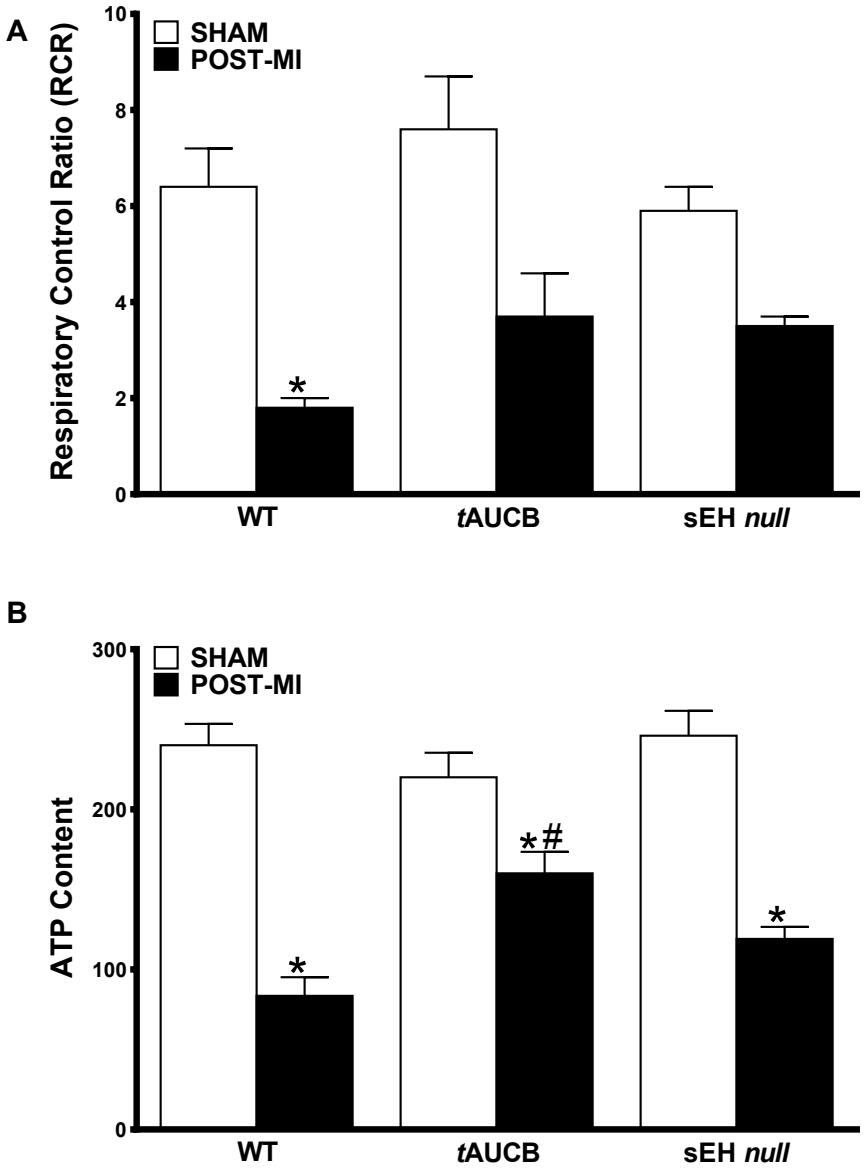

Figure 2.7: sEH suppresion preserve mitochondrial respiration. (A) Hearts from WT, tAUCB snd sEH null groups were sampled 7 d post-MI. Hearts were homogenized and mitochondria were isolated using differential centrifugation. Respiration in isolated mitochondria was measured using Clark-electrode based chamber connected to oxygraph. Rates of respiration are expressed as Respiratory Control Ratio (RCR). (B) ATP content was measured in the non-infarct region of the heart by a colorometric-based assay. Values are represented as mean ± S.E.M. N=4. Significance was $P<0.05$, *significantly different from its control sham, #significantly different from WT post-MI.

2.3.4 Inhibition of sEH and cardiac energy metabolism

Ex vivo working hearts were used to investigate the effect of sEH inhibition on energy metabolism following MI. In the absence of insulin, the rates of glucose oxidation were almost similar between the experimental groups (Figure 2.8A). In response to insulin, all sham hearts showed a significant increase in glucose oxidation. However, only hearts from *t*AUCB treated or sEH *null* mice demonstrated a significant response to insulin following MI (Figure 2.8A). Fatty acids are the primary energy substrate in the heart, and fatty acid β-oxidation is closely coupled with glucose oxidation via the Randle cycle. While the rate of palmitate oxidation was unaltered in the absence of insulin, the rate significantly decreased in all sham hearts after adding insulin. Damage from MI correlated with decreased basal palmitate oxidation in WT hearts compared to sham-operated mice but was not altered in hearts from *t*AUCB treated or sEH *null* mice. Moreover, palmitate oxidation was not altered after adding insulin in WT hearts post-MI (Figure 2.8B). Collectively, these data suggest that sEH inhibition preserved the cardiac response to insulin (i.e., insulin sensitivity) following MI.

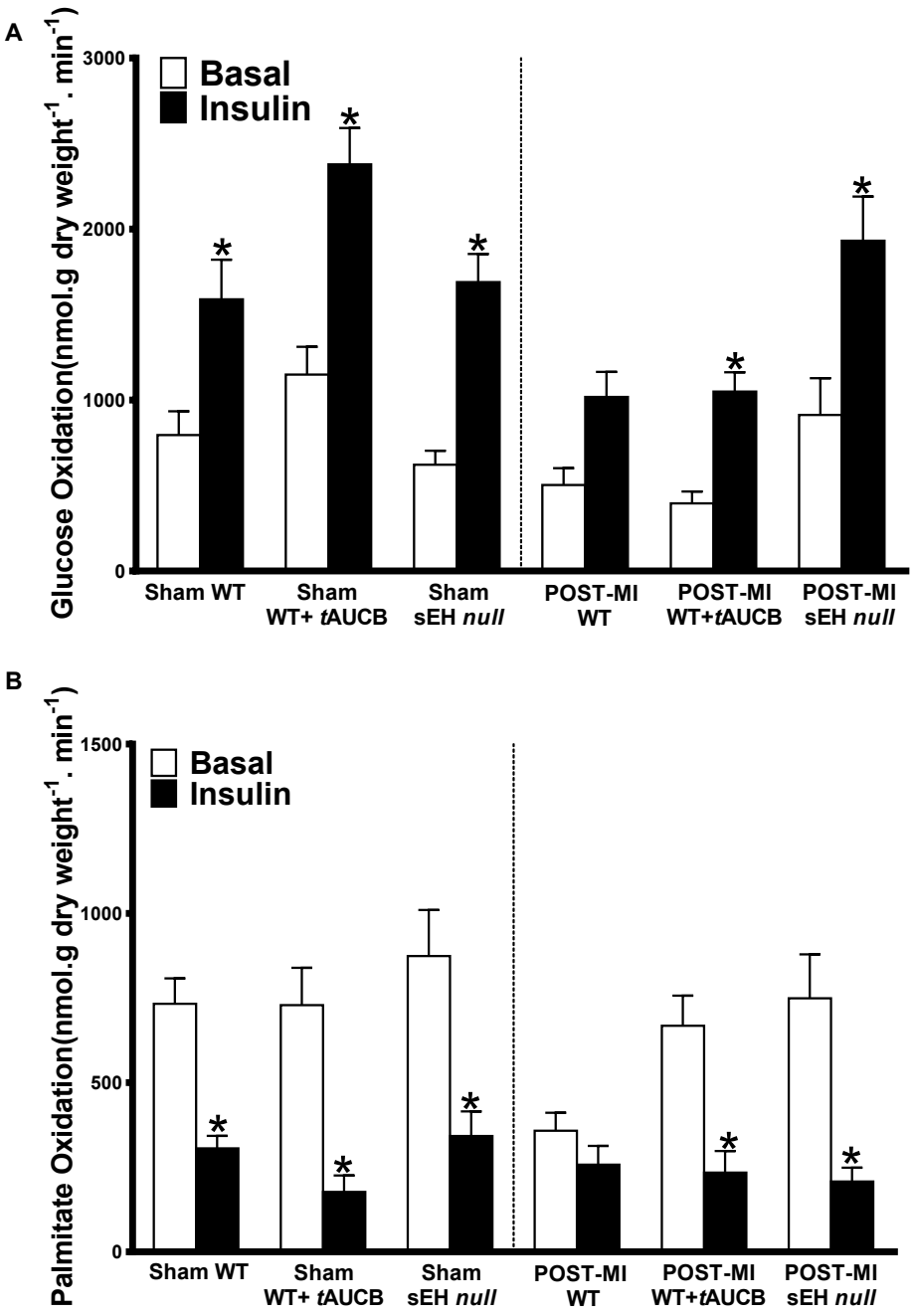

Figure 2.8: The effect of sEH suppression on cardiac metabolism. Increased insulin sensitivity of oxidative metabolism (A) Glucose oxidation and (B) palmitate oxidation in isolated working heart model in tAUCB and sEH null groups. Values are represented as mean ± S.E.M. N=6. Significance was $P<0.05$, *significantly different from respective basal oxidation.

2.4 DISCUSSION

This study demonstrates that both pharmacological and genetic approaches to inactivate sEH preserve mitochondrial and cardiac function following ischemic injury. Inhibition of sEH maintained cardiac insulin sensitivity post-MI. Restoration of cardiac insulin sensitivity associated with inactivation of sEH suggests that the injured region of myocardium is undergoing robust structural and functional recovery. Ischemic insult did not affect mitochondrial structure and protein content in non-infarct regions but dramatically reduced mitochondrial function, which was prevented by inhibition of sEH. Accumulation of aberrant mitochondria triggers further dissemination of injury on intracellular structures and eventually, leads to cardiomyocyte death. Our results demonstrate a key role of protecting mitochondrial function in mediating protective effects associated with inactivation of sEH.

Myocardial ischemia occurs when blood supply is stopped to the heart as a result of vascular blockage or injury resulting in a state of energy starvation (195). There are numerous detrimental consequences such as the development of mitochondrial crisis associated with defective cardiac metabolism eventually leading to heart failure (195-196). A large body of evidence has demonstrated a positive correlation between cardiac dysfunction in a failing heart attributable to decreased mitochondrial respiration rates (196). In this study we used a LAD occlusion model to replicate a condition of MI caused by permanent occlusion of the coronary

artery (197). As expected, ligated WT mice showed a decrease in LV systolic function and marked reduction in cardiac contractility, which was preserved in *t*AUCB treated and sEH *null* mice. It has been well documented that sEH inhibition increases the levels of endogenous EETs by suppressing their enzymatic degradation through sEH (47-48). The ratio of EETs to DHETs in mice is elevated in the plasma of *t*AUCB treated (48) and sEH *null* mice (142, 176). Previously published studies demonstrate genetic deletion of sEH gene or direct pharmacological inhibition of sEH activity produces a robust cardioprotective effect (49-52). This was confirmed by the cardioprotective effect of the sEH inhibitors AEPU and AUDA in the inhibition of hypertrophy in murine hearts after 3 weeks of thoracic aortic ligation (TAC) (52). Merabet et al used a rat LAD ligation model to show delayed treatment with sEH inhibitor (AUDA) preserves LV systolic function at early 3 days and late 42 days time points (41). Similar to our study, preservation of diastolic function was not observed at the early time point but only 42 days post MI (49). sEH suppression either pharmacologically or genetically produce a cardioprotective effect in mice exposed to 40 minutes of ischemia and 2 hours of reperfusion. This effect was observed when sEHi (AUDA-BE) was administered before or at the end of ischemia (169). Similar results were obtained in vitro when ventricular cardiomyocytes from neonatal mice were exposed to 90-min of oxygen glucose deprivation followed by 180-min of glucose-replete medium to stimulate IR. sEH inactivation using AUDA or 4-PCO in cultured cells enhances cell viability and tolerance against IR injury

(198). In this context, our data demonstrated that both sEH inhibition and gene ablation mediate a significant cardioprotective effect through attenuating the MI-induced LV dysfunction. This was associated with a significant preservation of cardiac contractility as measured by EF and FS. sEH inhibition also decreased the LV remodeling that was observed in the WT post-MI group as dilated LVESD and LVEDD.

The data indicated that sEH suppression preserves mitochondrial efficiency in the non-infarct region of the LV maintaining a healthy pool of cardiac mitochondria correlating with better contractility and functionality. Increased efficiency will supply the heart with sufficient amounts energy in the form of high-energy phosphates. As ATP production is primarily carried out by oxidative phosphorylation, damage to the ETC will lead to cardiac dysfunction. The failing heart becomes unable to produce sufficient amount of ATP to meet its contractile energy requirements (199). Our data show a decrease in ETC enzymatic function, respiratory control ratio and ATP content in WT post-MI hearts demonstrating mitochondrial dysfunction. The decreased mitochondrial function occurred in the non-infarct region as TEM images showed preserved mitochondrial ultrastructure. This suggests that the decline in mitochondrial function occurred prior to any remodeling or significant protein damage in WT mice. Interestingly, mitochondrial protein levels and ultrastructure in the non-infarct region were similar in all groups, but inhibition of sEH prevented the loss of mitochondrial function. Ischemic injury in myocardium is known to decrease the activity of complex I of the

ETC due to damage to an essential subunit in complex I (195, 200). Complex I defect leads to electron leak and generation of ROS which will eventually cause further damage to distal ETC complexes such as Complex III and IV (195).

The inhibition of sEH triggers a protective mechanism(s) that preserves mitochondrial function within the heart. We have previously shown the beneficial effects of EETs and sEH inhibition toward mitochondrial function, which prevented the loss of mitochondrial membrane potential ($\Delta\Psi_m$) and promoted cell survival (119, 144, 151, 201). In a starvation model of cell injury EETs preserve ETC enzyme activities and mitochondrial protein content in HL-1 cells and neonatal cardiomyocytes (158). It was demonstrated that EETs preserve mitochondria via activation of autophagy - which may shift death pathways toward survival. This results in maintaining a healthier pool of mitochondria either through removal of damaged mitochondria or improvement of ETC (158). This was observed by the enhanced ETC enzyme activities, oxygen consumption and ATP content.

Preservation of post-MI insulin sensitivity following inhibition of sEH supports the notion of better mitochondrial efficiency and cardiac function. The normal heart can easily switch substrate utilization to meet energy requirements according to changes in hormonal levels or substrate availability (79). Insulin sensitivity represents the responsiveness of insulin receptors and downstream signaling in insulin-responsive tissues (202). Early stages of heart failure have been associated with significant reductions in insulin

sensitivity and consequently, compromised glucose homeostasis (203). Luria et al demonstrated the role of sEH suppression in improving systemic insulin sensitivity and glucose homeostasis, where insulin sensitivity was preserved in both sEH *null* and *t*AUCB treated mice on a high fat diet (202, 204). They showed that sEH suppression stimulates insulin signaling in adipose tissue and liver due to activation of IRS-1 and PI3K (202). In the current study, we provide evidence that the cardic response to insulin was blunted 7 days post-MI, which was preserved following sEH inhibition. Restoration in insulin sensitivity reflects preservation of mitochondrial function, which is supported by studies demonstrating the association between cardiac insulin resistance and decreased mitochondrial function (205-209). Cardiac dysfunction in both mouse models and human hearts of individuals with type 2 diabetes caused by systemic insulin resistance have significant mitochondrial defects including decreased mitochondrial respiration and ATP production (205-207). Similarly, deletion of the insulin receptor in mice to develop insulin resistance decreases cardiac contractility, which is associated with a reduction in ATP production and mitochondrial respiration (208). A transverse aortic constriction model of heart failure produces cardiac insulin resistance leading to systolic dysfunction and exacerbation of contractile dysfunction (209). Therefore, restoring the insulin sensitivity by sEH suppression can be associated with the better mitochondrial efficacy demonstrated in our results by the enhanced ATP production and respiratory control ratio in a myocardial ischemia model.

The PI3K-Akt signaling pathway has a role in regulating insulin signaling, whereby phosphorylation of PI3K and Akt activate downstream mediators of the insulin cascade, including GLUT4 translocation, enhancing glucose metabolism (210-212). Preserved insulin sensitivity, observed in sEH *null* and *t*AUCB-treated mice on a high fat diet, was associated with activation of IRS-1-PI3K-Akt axis in the liver and adipose tissue (202, 204). It has been well established in previous studies that EET-mediated signaling involves activation of PI3K-Akt pathways limiting ischemia-reperfusion injury (142, 152, 155, 213). In vitro activation of the PI3K-Akt pathway was observed in EET-treated BAECs (152). Isolated murine hearts exposed to IR also demonstrated EET-mediated activation of PI3K. These results were consistent in sEH null mice (142), as well as, mice hearts perfused with EETs (155) or sEHi (BI00611953) (151). Inhibition of PI3K or Akt results in shutting down the insulin cascade and is considered a primary cause in the development of insulin resistance (210-212). Thus, activation of cardiac metabolism from one side and suppression of loss of mitochondrial function post-MI from another collectively result in promoting repair of myocardial structures and function. Activation of the PI3K-Akt pathway can be a potential mechanism by which sEH inhibition exerts its action.

In summary, we demonstrated pharmacological inhibition or genetic deletion of sEH mediates cardioprotective events through maintenance of mitochondrial efficiency. Our data show attenuation of sEH prevents systolic dysfunction following ischemic injury by preserving the mitochondrial pool in

the non-infarct region of the heart. Furthermore, inhibiting sEH preserved insulin sensitivity in post-MI hearts reflecting more optimal functioning cardiac metabolism thereby suggesting there was activation of physiological recovery from ischemic insult.

CHAPTER 3

CONCLUDING REMARKS AND FUTURE DIRECTIONS

3.1 Concluding Remarks

In this thesis, we attempted to study the underlying mechanisms of cardioprotection mediated by sEH suppression. A large body of evidence has demonstrated several signaling pathways mediated by sEH suppression and EETs, however, the exact molecular mechanism(s) are poorly understood (45, 214). Investigating these mechanisms could facilitate the discovery of new therapeutic agents which can protect the heart against IHD and ischemic injury.

Evidence has demonstrated the crucial role of CYP epoxygenases in the CVS. EETs, which are metabolites of AA through CYP epoxygenases, are studied for their cardioprotective effects through several mechanisms (45). Several approaches are used to study the cardioprotective effects of EETs; such as genetically overexpression of CYP2J2 to increase EETs production or deletion of sEH to inhibit EETs degradation. Pharmacological EET mimetics and sEH inhibitors are synthesized for the same reason.

The data in the current thesis demonstrate both pharmacological methods (sEHi) and genetic method (sEH null) to to enhance EET level. In vivo induction of myocardial ischemia was applied by LAD ligation in experimental mice. The study presented in this thesis demonstrated that sEH suppression, either pharmacologically or genetically, confer a cardioprotective effect against ischemic injury. The effect of sEH suppression in preservation of cardiac function was clearly demonstrated by this study and others. We

provided evidence for the first time that EET-mediated cardioprotective effects involve preservation of mitochondrial efficiency. In spite of the preservation of mitochondrial ultrastructure and protein content in the non-infarct region of the LV after ischemic injury, significant decrease in mitochondrial function was observed. We found that sEH suppression was associated with better function of the mitochondrial respiratory enzymes in the non-infarct region after exposure to ischemic injury. Our group, as well as others have previously shown that EET and sEH inhibition is associated with preservation of mitochondrial function (119, 151, 215). Furthermore, we observed a significant enhancement in mitochondrial respiration in isolated mitochondria and higher ATP content in sEH suppressed mice exposed to ischemic injury. Remarkably, inactivation of sEH results in restoration of cardiac insulin sensitivity post-MI which indicates structural and functional recovery of the cardiomyocytes. Evidence showed that EET play a crucial role in activation PI3K/Akt pathway in the cardiomyocytes (142, 152, 155, 213). We suggested that sEH inactivation could result in activation of the PI3K/Akt pathway, which in turn enhances insulin sensitivity. Preserved insulin sensitivity might be linked with the better mitochondrial efficiency through enhancing mitochondrial respiration and ATP production.

We have previously described the involvement of mitochondria in cardioprotection mediated by sEH activation and EET following ischemic injury (151, 155, 158, 178). In accordance with the previous reports, we found that sEH suppression confer its cardioprotective effect through preservation of

mitochondrial activity. Collectively, we were the first to report the preservation of mitochondrial efficiency and metabolic state in the non-infarct region of the heart after seven days of global ischemia. Taken together, these data provide new insights in the mechanisms underlying the cardioprotective effect mediated by sEH inactivation.

3.2 Future Directions

Evidence and data from the present thesis demonstrate that sEH suppression mediates cardioprotective effect in mice exposed to myocardial ischemia. Most importantly, sEH suppression results in preservation of a healthy pool of mitochondria in the non-infarct region of the heart. However, further experiments should be conducted to better understand the molecular mechanisms involved in those effects.

1-Experiments to investigate the key mediators linking sEH inactivation and mitochondrial efficiency:

Future studies should include investigating the different signaling pathways that can be associated with cardioprotective effects observed in hearts following sEH suppression. Validation of the involvement of PI3K/Akt signaling pathway can be assessed by measuring the PI3K activity and the phospho-Akt content in the tissues. Our group previously demonstrated that PI3Kα plays an important role in the EET-mediated cardioprotection (155). Therefore, further investigation of the PI3k isoforms involved can be studied using targeted deletion or co-treatment with specific PI3K inhibitors. In

addition to that, sEH catalyzes the hydrolysis of many epoxides; as EETs, EpOMEs and others. Therefore, further studies are needed to investigate the key mediators of the cardioprotective effects seen by sEH suppression.

2- Experiments to investigate the effect of using sEH inhibitors post-MI:

In the present study we added tAUCB (sEHi) 4 days before MI which is not a clinically optimal treatment protocol. Future studies are needed to investigate the effect of sEHi at different time points post-MI. This will assist in exploring a more clinically relevant approach to understanding the protective effect toward mitochondrial function and the role in limiting the adverse effects caused by cardiac remodeling.

3- Experiments to investigate the effect of sEH suppression on mitochondria of aged mice

Cardiovascular diseases are a leading cause of morbidity and mortality in elderly individuals, where aging increases the susceptibility of the heart to stress factors, like ischemia-reperfusion, causing lethal outcomes. Therefore, targeting strategies that reduce mitochondrial damage is important. The overall goal is to investigate the protective role of EETs in the aged heart. Molecular mechanisms involved in the regulation of mitochondrial quality control in aged hearts need to be investigated which may identify potential therapeutic targets that prevent and reduce injury.

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