University of Alberta

Mechanisms of epoxyeicosatrienoic acid-induced cardioprotection

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Fall 2012
Edmonton, Alberta

Dedication

I dedicate this thesis to my loving parents and grandparents.

ABSTRACT

Ischemic heart disease remains a major cause of illness, disability and death worldwide; as such there remains a need for novel pharmacological agents that protect against myocardial ischemia reperfusion (I/R) injury. Epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acid, which have demonstrated protective properties against cardiac I/R injury. However, the exact mechanism(s) of cardioprotection remains unknown. The work presented in this thesis investigated potential mechanisms underlying EET-induced cardioprotection in young and aged animal models.

I/R injury was examined in the Langendorff isolated perfused heart model. Key results demonstrate that preventing EET-removal by inhibiting soluble epoxide hydolase (sEH), with sEH inhibitor (sEHi) or targeted deletion (sEH null), improved left ventricular functional (LVDP) recovery and reduced injury following I/R. The improved postischemic recovery was blocked by phosphoinositide 3-kinase (PI3K) inhibitors. Data demonstrated that increased B-type natriuretic peptide (BNP) mRNA and protein expression in sEH null and EET-perfused wild type (WT) hearts. Moreover, perfusion with BNP receptor antagonist attenuated the improved postischemic LVDP recovery. Increased expression of activated PKCε and Akt were found in WT hearts perfused with either 11,12-EET or BNP. In addition, treatment with the PI3K inhibitor (wortmannin) abolished improved postishcemic functional

recovery in 11,12-EET treated hearts but not of BNP treated hearts. In the final study, data demonstrated that EET-induced cardioprotection remains effective in aged mice. Importantly, aging is a major risk factor for development of IHD and increases susceptibility to I/R injury. Furthermore, many cardioprotective strategies become ineffective in aged animals. Interesting data from aged mice demonstrated that the cardiomyocyte specific over-expression of CYP2J2 (CYP2J2 Tr) reduces the cardioprotective response. The loss of cardioprotection in aged CYP2J2 Tr was attributed to increased linoleic metabolite (DiHOME), oxidative stress and decreased protein phosphatase 2a activation. Moreover, all these effects and loss of cardioprotection in aged CYP2J2 Tr mice can be prevented by sEHi.

In summary, results from this thesis clearly demonstrate EETs protect the heart against I/R injury and the protective effects are mediated through BNP and PI3K pathway. In addition, we demonstrate the effectiveness of EETs and sEHi in protecting the heart against I/R injury in aged animals.

AKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Dr. John Seubert for providing me the opportunity to pursue my graduate studies under his supervision. Without his guidance, support and encouragement, this thesis would not have been possible. He was always present whenever I needed him to discuss new findings, to discuss new experiments and for general advise.

I would like to thank my committee members, Dr. Dion R. Brocks and Dr. Jason R. B. Dyck for their continuous guidance, encouragement and insightful comments throughout my graduate studies. I would also like extend my special thanks to Dr. Ayman El-Kadi and Dr. Mavanur Suresh for their co-operation in some part of my research.

I would like to acknowledge my previous lab colleagues Dr. Sri Nagarjun Batchu and Dharmendra Katragadda for teaching me very first steps in research and for being with me in good and bad times during my graduate studies. I would like to thank my previous and present lab members; Dr. Yunfang Zhang, Mohamed Abukhashim, Haitham El-Sikhry, Rawabi Qadi, Glenis Weib and Naser Alsaleh for their support and being such wonderful colleagues to work with.

Thanks to my mother, Shri Shantaben Chaudhary, and my father, Shri Rameshbhai Chaudhary, for always supporting me by all means, for always believing in me, and for raising me up to be the person I am today.

Thanks to my wife, Kruti Chaudhary, for her love and support, for her patience in listening to my complaints about experiments that do not work, and for her tolerance when I had to work late nights and weekends to finish my work.

I would like to thank my sisters, Kajal Chaudhary and Hetal Chaudhary, for their encouragement, love and support.

Thanks to all my friends Japan Trivedi, Sejal Dave, Mridul Jain, Charu Jain, Jigar Patel, Mranal Jain, Rhitika Bhansali, Sarthak Patel, Amit Jhala, Balbhadar Katoch and Pratik Arvadia for their constant support.

Thanks to the funding agencies for providing financial support during my graduate studies: Alberta Innovates Health Solutions and Heart and Strike Foundation of Canada.

I would like to extend appreciation to the Faculty of Graduate Studies and Research and the Faculty of Pharmacy and Pharmaceutical Sciences of the University of Alberta for their assistance and support.

Thanks to everyone in the Faculty of Pharmacy and Pharmaceutical Sciences as well as in the Cardiovascular Research Centre. Thanks to all the administrative staff in the Faculty of Pharmacy and Pharmaceutical Sciences for always answering my questions and solving my problems.

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14,15-EEZE	14,15-epoxyeicosa-5(Z)-enoic acid
5-HD	5-Hydrocydecanoate
$\Delta\Psi m$	Mitochondrial membrane potential
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ACEi	Angiotensin converting enzyme inhibitor
AEPU	1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea
Akt	Protein Kinase B or PKB
AMPK	cAMP activated protein kinase
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptides
ANT	Adenine nucleotide translocator
AUC_t	Area under concentration time curve
AUDA	12-(3-adamantan-1-yl-ureido)-dodecanoic acid
AUDA-nBE	12-(3-adamantan-1-yl-ureido)-dodecanoic acid *n*-butyl ester
BK_{Ca} channels	Large conductance Ca^{2+}-activated K^+ channels
BNP	B-type or brain natriuretic peptides
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CAV	Caveolin
CNP	C-type natriuretic peptides
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase or PGHS

CRE	cAMP-response element
CVD	Cardiovascular disease
CYP	Cytochrome P450
CYP2J2 Tr	Mice with cardiomyocyte specific overexpression of CYP2J2
DCM	Dilated cardiomyopathy
DHET	Dihydroxyeicosatrienoic acid
DiHOME	Dihydroxyoctadecenoic acid
DNP	Dendroaspis natriuretic peptides
EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
EpOME	Epoxyoctadecenoic acid
Erk	Extracellular signal-regulated kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GSH	Glutathione reduced
GSSG	Glutathione oxidized
GSK3β	Glycogen synthase kinase-3β
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
IHD	Ischemic heart disease
IPC	Ischemic preconditioning
I/R	Ischemia reperfusion
JAK	Janus kinase
Lck	Lymphocyte-specific protein tyrosine kinase

LDH	Lactate dehydrogenase
LOX	Lipoxygenase
LVDP	Left ventricular developed pressure
MAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
mEH	Microsomal epoxide hydrolase
mitoK_{ATP} channel	Mitochondrial ATP-sensitive K^+-channel
mPTP	Mitochondrial permeable transition pore
MS-PPOH	N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide; CYP epoxygenase inhibitor
NHE	Sodium-hydrogen exchanger
NO	Nitric oxide
Nppb	Prepro-BNP
NPR	Natriuretic peptide receptors
P70S6K	p70S6 Kinase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PDE	Phosphodiesterase
PDK	Phosphoinositide-dependent protein kinase
PG	Prostaglandins
PGHS	Prostaglandin H_2 synthase
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3'-OH kinase
PLA2	Phospholipase A2

PKA	Protein Kinase A
PKB	Protein Kinase B or Akt
PKC	Protein kinase C
PKG	Protein kinase G
POC	Post-conditioning
PP2A	Protein phosphatase 2A
PTEN	Protein phosphatase and tensin homolog
qPCR	Quantitative real-time PCR
rBNP	Recombinant B-type or brain natriuretic peptides
RIC	Remote ischemic conditioning
RISK	Reperfusion induced salvage kinase
ROS	Reactive oxygen species
SAFE	Survivor activating factor enhancement
sarcK_{ATP}	Sarcolemmal K_{ATP} channels
sEH	Soluble epoxide hydrolase or EPHX2
sEHi	Soluble epoxide hydrolase inhibitor
sEH null	Mice with targeted deletion of soluble epoxide hydrolase or sEH KO
SEM	Standard error of mean
SERCA	Sarcoplasmic reticulum calcium ATPase
SNP	Single nucleotide polymorphisms
STAT	Signal transducer and activator of transcription
SWOP	Second window of protection
$t_{1/2}$	Half life
t-AUCB	*trans*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid

TB/Tet^{5}	Terrific broth medium containing 5 µg/ml of tetracycline
TB/Tet^{5}/Chlor^{34}	Terrific broth medium containing 5 µg/ml of tetracycline and 34 µg/ml chloramphenicol
TTC	2,3,5-triphenyltetrazolium chloride
TUPS	1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea
TX	Thromboxanes
VDAC	Voltage dependent anion channel
WHO	World health organization
WT	Wild type

Chapter 1

Introduction

1.1 Ischemic heart diseases

Cardiovascular disease (CVD) is one of the leading causes of mortality in Canada and other Western countries, accounting for approximately 30% of overall deaths.[1-3] According to the World Health Organization (WHO), CVD will be the leading cause of death by 2030 and could cause death of more than 23 million people in the world.[3] While CVD significantly impacts human life, it also causes large economic burden on national healthcare systems.[4] Therefore, a thorough effort is needed to understand, prevent and treat CVD in order to limit its consequences. More than 50% of CVD related deaths can be attributed to ischemic heart diseases (IHD).[1-2] IHD, also known as coronary artery disease (CAD), occurs when both the blood flow and the oxygen supply to the heart are restricted. In most of the cases myocardial ischemia occurs subsequent to the formation of the atherosclerotic plaque in the coronary artery. Reduction in the coronary blood flow due to atherosclerotic plaque causes inadequate myocardial oxygen supply which is necessary for maintaining the normal heart function (Fig 1). Myocardial ischemia, within few minutes, causes significant damage to the cardiac muscle and impairment of heart function that can lead to permanent disability as well as death. Prevention of the damage to cardiac muscle following the ischemic injury is the primary focus of the work presented in this thesis.

Current therapy for myocardial ischemia primarily focuses on re-establishing the blood flow to ischemic myocardium to prevent further damage.[5] Blood flow to the ischemic zone can be re-established by drugs (thrombolytic drugs), surgery (bypass grafting) or non-surgical methods (angioplasty). Other treatments target improvement in hemodynamic parameters and reducing oxygen consumption by the heart muscle. Drugs, such as nitroglycerin and calcium channel blockers, transiently dilate the arteries and increase the blood supply to the heart. On the other side, drugs like β-blockers, calcium channel blockers and angiotensin converting enzyme (ACE) inhibitors decreases the workload of the heart by dilating the blood vessels, decreasing the blood pressure or relaxing the cardiac muscle. In addition, treatment with cholesterol lowering agents can further reduce the risk of reoccurrence of the coronary atherosclerosis. All these treatments can reduce the imbalance between oxygen consumption and oxygen supply, reduce the chances of reoccurrence of the event and improves the hemodynamic parameters; however, they do not directly prevent the cell death that occurs during ischemia and reperfusion period. Current basic science work mainly focuses on understanding the mechanisms of cell death and identifying targets for prevention of myocardial cell death following ischemic injury. Identifying the targets can be helpful in designing the novel drugs that directly modulate the endogenous signaling pathways, prevent the cardiomyocyte death and improve the outcome following myocardial

ischemia. The focus of this thesis is to study the cardioprotective effects of cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acid and the mechanisms underlying the cardioprotection. The introduction will provide an overview of mechanisms of ischemia reperfusion (I/R) injury, cardioprotective strategies and signaling pathways involved in cardioprotection, mainly the pathways activated by CYP epoxygenase metabolites of arachidonic acid.

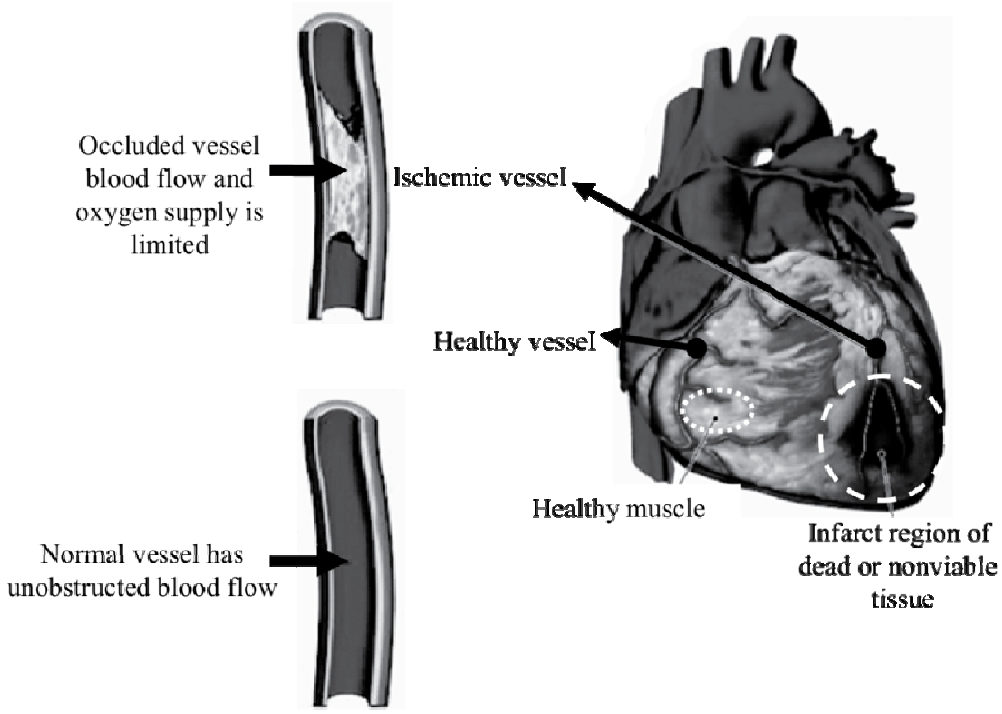

Figure 1.1 Schematic diagram representing the coronary artery disease. Plaque formation begins with cholesterol adhering to walls of coronary vessels. As plaque becomes larger artery expands and lumen of the vessel decreases. Formation of blood clot causes occlusion of vessel by a thrombotic plug that results myocardial ischemia and development of infarct.[6]

1.2 Myocardial ischemia reperfusion (I/R) injury.

The heart is a dynamic organ that has a very high-energy demand. This energy is supplied in the form of ATP, which is produced mainly by mitochondria (>95%) through oxidative phosphorylation, glycolysis and citric acid cycle.[7-8] Continuous blood flow is required to supply enough oxygen to produce ATP. Under normal conditions, without CAD, coronary blood flow increases in response to stress, such as exercise, to maintain oxygen supply to the cardiac muscle.[9] Under pathophysiological conditions, such as in the presence of atherosclerotic plaque in the coronary artery, reduction in blood supply to the heart leads to development of ischemia.[10] Myocardial ischemia causes significant alterations in cardiac energy production, Ca^{+2} overload and activation of proteases and phosphatases leading to cell death. Myocardial cell death varies depending upon the duration and severity of ischemia.[11] The effects are initially reversible; however, if oxygen is deprived for an extended period of time they become more severe and irreversible.[11] Therefore, immediate re-introduction of the blood flow to the ischemic myocardium is vital.[12-13] Conversely, the process of restoring blood flow can itself cause myocardial injury, termed reperfusion injury. Reperfusion injury was first postulated in 1960 where Jennings et al., reported histologic features of reperfused ischemic canine myocardium.[14]

1.2.1 Ischemic injury

Major cellular events occur following I/R trigger a series of reactions leading to irreversible injury. The cascade of events begins during ischemia where reduced availability of molecular oxygen and metabolic substrates results in decreased ATP synthesis. This is due to the inhibition of the respiratory chain and oxidative phosphorylation.[15] In response to low levels of oxygen, the cell switches from aerobic to anaerobic metabolism for ATP synthesis. This leads to the accumulation of inorganic phosphate, lactate and protons.[15-16] Accumulation of protons causes activation of the sodium-hydrogen exchanger (NHE) and $Na^{+}HCO^{3-}$ transporter thereby increasing intracellular Na^{+}.[15-16] Decreased ATP levels will lead to the failure of ATP-dependent ion pumps ($Na^{+}K^{+}$ATPase) that will prevent the exclusion of Na^{+} from the cell and lead to increase in intracellular Na^{+}. High intracellular Na^{+} will lead to increases intracellular Ca^{2+} through $Na^{+}Ca^{2+}$ exchange.[17] A net inward flow of Ca^{+2} and dysfunctional intracellular Ca^{+2} stores (SERCA) causes Ca^{+2} to overload the cell resulting in adverse effects like inhibiting contractility.[16] Moreover, increased intracellular Ca^{2+} leads to activation of Ca^{2+}-dependent proteases and phospholipases that begin to breakdown cellular components. Increased intracellular Ca^{2+} further causes mitochondrial Ca^{2+} overload and dysfunction of mitochondria. Mitochondrial dysfunction and breakdown initiates release of toxins and apoptotic factors that ultimately leads to cell death and development of stable infarct (Fig 1.2).

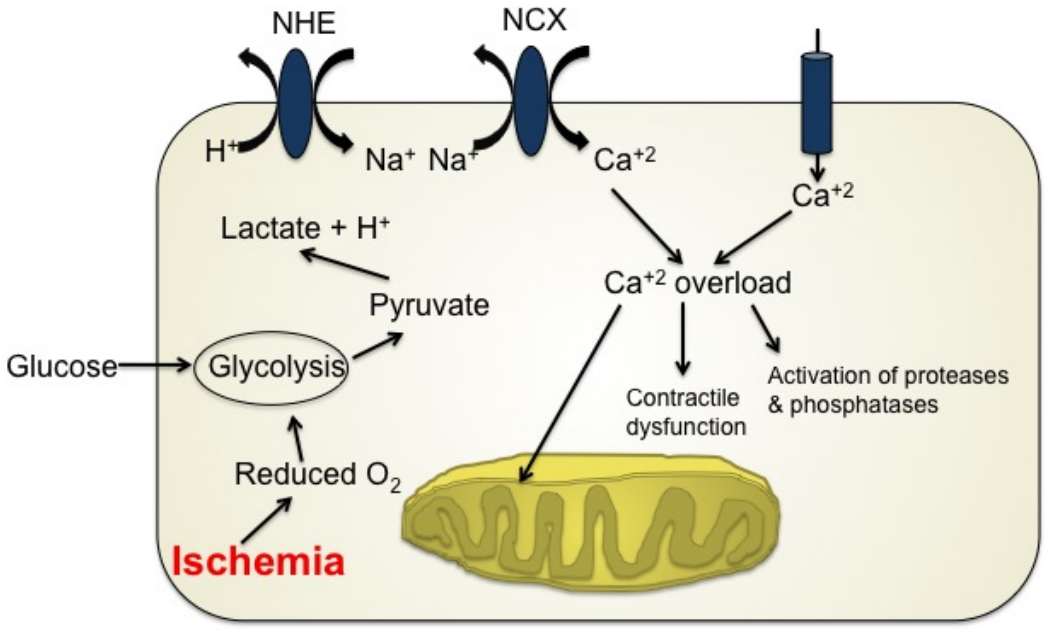

Figure 1.2 Schematic diagram demonstrating ischemic injury. In response to decreased oxygen during ischemia, the cell switches from aerobic to anaerobic metabolism for energy production. Anaerobic metabolism results in accumulation of lactate and H^+ which further leads to a series of events causing increased intracellular Ca^{+2}. Increased intracellular Ca^{+2} further causes mitochondrial dysfunction, contractile dysfunction and activation of proteases and phosphatases, ultimately leading to cell death.

1.2.2 Reperfusion injury

Braunwald and Kloner regarded myocardial reperfusion as a "double-edged sword".[18] While immediate reperfusion following an ischemic event is essential for salvage of the ischemic myocardium, re-introduction of flow comes with a great risk of an expansion or worsening of the events initiated during an ischemic event.[16] Oxygen free radicals, Ca^{2+} overload, neutrophils-endothelium interaction and apoptosis are thought to be the major mediators of reperfusion injury.[15, 19] The sudden re-introduction of oxygen causes re-energization of the mitochondria and reactivation of the electron transport chain (Fig 1.3).[20] The electrons swiftly flow into the mitochondria but cannot be used by the mitochondria for oxidative phosphorylation because of a loss of cytochrome oxidase and dismutase enzymes activity.[20] This leads to the formation of free radicals such as O_2^- and H_2O_2. Free radicals generated during reperfusion from injured mitochondria after an ischemic insult lead to cytoskeleton damage, increased lipid peroxidation, increased cell permeability and altered membrane potential ($\Delta\Psi m$).[20] Alteration in membrane potential by free radicals, increased levels of calcium leading to further injury and swelling of inner mitochondrial membrane all trigger the opening of the mitochondrial permeable transition pore (mPTP)[21-22] Opening of mPTP is associated with irreversible necrotic cell death in cells that sustained significant injury during ischemia; however, some cells display hallmarks of apoptosis after reperfusion injury.[23]

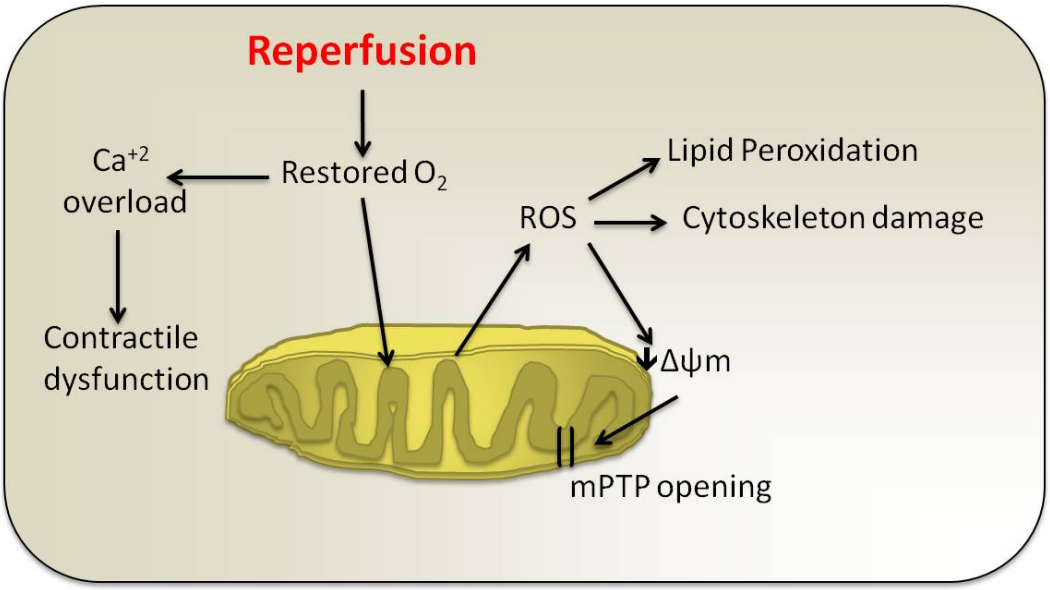

Figure 1.3 Schematic diagram demonstrating reperfusion injury. Sudden reintroduction of oxygen following ischemia causes expansion of the damage occurred during ischemia. Reperfusion further increase in intracellular Ca^{+2} and also generates reactive oxygen species (ROS). Increased intracellular Ca^{+2} and ROS results in mitochondrial Ca^{+2} overload, mitochondrial dysfunction, opening of mPTP, lipid peroxidation and cytoskeleton damage finally leading to further increase in cell death.

1.3 Protection against I/R injury

1.3.1 Ischemic preconditioning

About 25 years ago, Murry et al. reported that a series of short periods of target tissue ischemia before the sustained ischemia substantially reduced infarction, termed ischemic pre-conditioning (IPC).[24] To date, IPC is the most effective and reproducible technique limiting I/R injury thought to involve induction of endogenous protective mechanisms. Effectiveness of IPC to protect the heart against I/R injury has now been demonstrated in various species, including mouse [25], rat [26], pig [27], sheep [28] and human [29]. Originally, the effect of IPC was analyzed by measuring the reduction in the infarct size.[24] Nevertheless, IPC has also been demonstrated to be beneficial in reducing arrhythmias [30-31], decreasing lactate dehydrogenase (LDH) release and to improve postischemic left ventricular function [32].

IPC triggers endogenous protective mechanisms in response to episode of acute ischemia–reperfusion.[33] The brief periods of ischemia and reperfusion, which comprise the IPC stimulus, elicit two distinct windows of cardioprotection: first, termed classical IPC and a second window of protection (SWOP). Effects of classical IPC appear immediately and wane after 2–3 h while the SWOP appears 12–24 h later and lasts 2–3 days.[33] This suggests that the classical IPC results due to immediate action of the signaling molecules while SWOP occurs due to synthesis

and release of new molecules. Interestingly, classical IPC, in dogs, remains ineffective if the index ischemic period is greater than three hours.[24] IPC requires an intervention be applied before the onset of the index myocardial ischemia, as such clinical application of IPC has been largely constrained to specific conditions in which the ischemic insult can be anticipated, for example cardiac surgery. Research has focused on investigating possible signaling mechanisms underlying IPC with the intention of identifying pharmacological targets. Despite the large amount of research investigating this phenomenon, the exact mechanism(s) that underlie IPC are still not fully understood. Some of the key proteins thought to be involved in transmitting the IPC signal, include phosphatidylinositol-3'-OH kinase (PI3K), protein kinase B (PKB or Akt) and protein kinase C (PKC).

1.3.2 Post-conditioning

While IPC is clearly a powerful cardioprotective treatment, a similar protocol of episodes of sub-lethal ischemia and reperfusion administered subsequent to the index ischemia produces a cardioprotective effect, called post-conditioning (POC).[34] Similar to IPC, POC is effective in protecting the heart against I/R injury in various species including rat [35], mouse [36], rabbit [37], pig [38-39], dog [34, 40] and human [41]. Zhao et al first reported that post-conditioning of canine hearts with three cycles of 30sec coronary artery re-occlusions and 30sec reperfusion protected the

heart and improved the outcomes following ischemic injury.[34] Several beneficial effects of POC include reduction in infarct size, reduced edema, reduced neutrophil accumulation, reduced apoptotic cell death and improved endothelial function.[34] The ability of POC to reduce myocardial injury suggests prevention of damage triggered during reperfusion can greatly improve the outcome of myocardial I/R injury. Similar to IPC, POC protects the heart by attenuating the effect of adverse mediators from reperfusion injury such as oxidative stress, calcium accumulation, inflammation and mPTP opening.[39]

1.3.3 Remote ischemic conditioning

IPC and POC are cardioprotective techniques that involve direct conditioning of the heart. Interestingly, significant cardioprotection can be achieved by applying the brief episodes of non-lethal ischemia and reperfusion to an organ or tissue remote from the heart; remote ischemic conditioning (RIC).[13, 42] The conditioning could be performed before ischemic event, during the ischemic period or at the reperfusion.[13, 42-43] RIC has been demonstrated to have protective effects in the heart against acute ischemia–reperfusion injury in animal models as well as in human.[42-44] Many of the signaling pathways underlying RIC induced myocardial protection are similar to IPC and POC.[42] RIC mediated cardioprotection involves activation of K_{ATP} channels as well as kinases such as Akt and extracellular signal-regulated kinase (Erk1/2).[42, 45]

However, it is of great interest to understand the mechanisms by which remote organ signals and protect the heart against I/R injury. Clearly, further studies are required to dissect the underlying mechanism.

1.3.4 Pharmacological conditioning

IPC, POC and RIC are non-pharmacological conditioning strategies that protect the heart against I/R injury. The pharmacological conditioning refers to the use of chemical or biological agents that target specific pathways that protect the heart. Pharmacological conditioning can effectively prevent or reduce myocardial infarct size and improve the post-ischemic heart function when administered before or after index ischemia.

Pharmacological agents such as adenosine [46-48], opioids [49-52], ACEi [53-54], bradykinin [55-56], erythropoietin [57], volatile anesthetics [58-59] and PKCε agonists [60] have been demonstrated to induce protection against I/R injury in animal models as well as in humans.[61] Adenosine has been demonstrated to play an important role in mediating the protective effects of POC [62-64] and studies have shown protective effects of adenosine and the adenosine receptor agonist against I/R injury.[46-48] On the other side, some animal studies as well as clinical trials have reported no benefit of adenosine against I/R injury.[65-66] Nevertheless, AMISTAD-II trial at-least demonstrated effectiveness of adenosine in decreasing infarct size following acute myocardial infarction and suggested the requisite of a larger clinical study

to study the protective effects of adenosine.[66] Preclinical studies using opioid receptor agonists, such as morphine (non-selective opioid receptor agonist), BW373U86 (selective δ-opioid receptor agonist) and U50-488 (selective κ-opioid receptor agonist), demonstrated protective effects of opioids against I/R injury.[49-52] Moreover, a small clinical study reported beneficial effects of morphine in combination with volatile anesthetics in patients undergoing coronary bypass grafting.[67] Several preclinical and clinical studies reported beneficial effects of volatile anesthetics when given at the onset of ischemia or at the time of reperfusion.[59, 68-69] Apart from these, various other chemically diverse pharmacological agents have demonstrated effective cardioprotective properties [61]; however, there is no drug available in the market that is approved for the treatment of myocardial infarction. Various molecular signaling pathways are involved in mediating protective effects of these pharmacological agents.

1.4 Mediators of cardioprotective signaling

IPC, POC and other conditioning strategies have been demonstrated to have cardioprotective effects and multiple survival pathways have been reported to play important role in mediating the cardioprotective effects. Such cardioprotective pathways include the reperfusion induced salvage kinase (RISK) pathway and survivor activating factor enhancement (SAFE) pathway.[70-72] These pathways,

activated in response to a number of different stimuli, ultimately lead to changes in growth and function. Various signaling molecules such as PI3K-Akt, Erk1/2, PKC, glycogen synthase kinase-3β (GSK-3β), endothelial nitric oxide synthase (eNOS), protein kinase G (PKG) and janus kinase and signal transducer and activator of transcription (JAK-STAT) are part of these cardioprotective signaling pathway.[70-72] This thesis will focus on the PI3K-Akt, GSK-3β, PKCε and natriuretic peptide.

1.4.1 Phosphoinositide 3-kinases (PI3Ks)- Protein kinase B (PKB or Akt)

Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes implicated in signal transduction involved in cell survival and growth.[73-74] There are three classes of PI3Ks: class IA/B, class II and class III. Class IA/B PI3K enzymes exist as a dimer formed by a p85 regulatory (alpha, beta or delta) and a p110 catalytic subunit (alpha, beta, delta or gamma).[73-75] Conversely, class II enzymes consists of a catalytic subunit without the regulatory subunit.[74] Structurally, class III PI3Ks are similar to class I with a catalytic and a regulatory subunit. Class 1A PI3K are activated by tyrosine kinase receptor activation, for example insulin growth factor-1 receptor [76]; whereas class 1B are activated by the βγ subunit of G-proteins and acts downstream of G-protein–coupled receptors.[75]

Effects of activated PI3K are mediated through a serine threonine kinase, Akt. Three isoforms of Akt are known; Akt1, Akt2 and Akt3.[77] Expression of Akt1 and Akt2 are normally found in all the organs including the heart; however, Akt3 is primarily expressed in brain and testis.[77] Activated class 1 PI3Ks convert PI(4,5)P2 to PI(3,4,5)P3 which then binds to the pleckstrin homology (PH) domain of the target proteins.[78] In response to the formation of PI(3,4,5)P3 by PI3Ks, protein containing PH-domain, such as Akt and phosphoinositide-dependent protein kinase 1 (PDK1), translocates to the plasma-membrane (Fig 1.4).[78-79] PDK1 phosphorylates Akt at threonine 308 that causes partial activation of Akt while phosphorylation of Akt at serine 473 by PDK2 results in full activation.[77-79] Akt can further phosphorylate and regulate a large number of diverse downstream proteins.[77] Hence, Akt is an important part in many signaling pathways regulating various cellular functions. PI3K induced Akt activation can be negatively regulated by the protein phosphatase, tumour suppressor protein phosphatase and tensin homolog (PTEN).[80] PTEN suppresses the Akt activation by catalyzing the opposite reaction to PI3K, i.e. de-phosphorylates PI(3,4,5)P3 to PI(3,4)P2.[80] Therefore, PTEN prevents the translocation of Akt to the plasma-membrane, suppresses Akt activation and inhibits PI3K-Akt signaling (Fig 1.4).

PI3K-Akt mediated events involve modulation of cell growth and proliferation, glucose homeostasis, postnatal brain development, heart

growth and cardiac hypertrophy, skin growth, adipogenesis, bone development and lipid metabolism in the mammary gland.[81-83] The effects of PI3K-Akt signaling are primarily mediated through downstream targets including GSK-3β, p70S6K, eNOS and Bcl-2 proteins (For example, BAD) (Fig 1.4).[77] An important function of Akt is to inhibit apoptosis.[84] Anti-apoptotic effects are mediated through Akt-induced inhibition of caspase 9, inhibition of pro-apoptotic factors and altering gene transcription factors associated with survival and cell death.[85] In regards to I/R injury, it has been demonstrated that the activation of PI3K-Akt pathway play important role in protection of ischemic myocardium.[86-87] IPC-induced activation of PI3K-Akt pathway, either before or after ischemia, protects the heart against I/R injury and reduces infarct size.[88-91] Moreover, POC has been demonstrated to increase Akt phosphorylation during reperfusion and protect the heart against I/R injury.[92-94] Similar to IPC and POC, many pharmacological agents including insulin [95], bradykinin [96], erythropoietin [97-98] and glucagon-like peptide 1 [99] has been demonstrated to protect the heart against I/R injury and all of these agents are known to activate PI3K-Akt pathway.

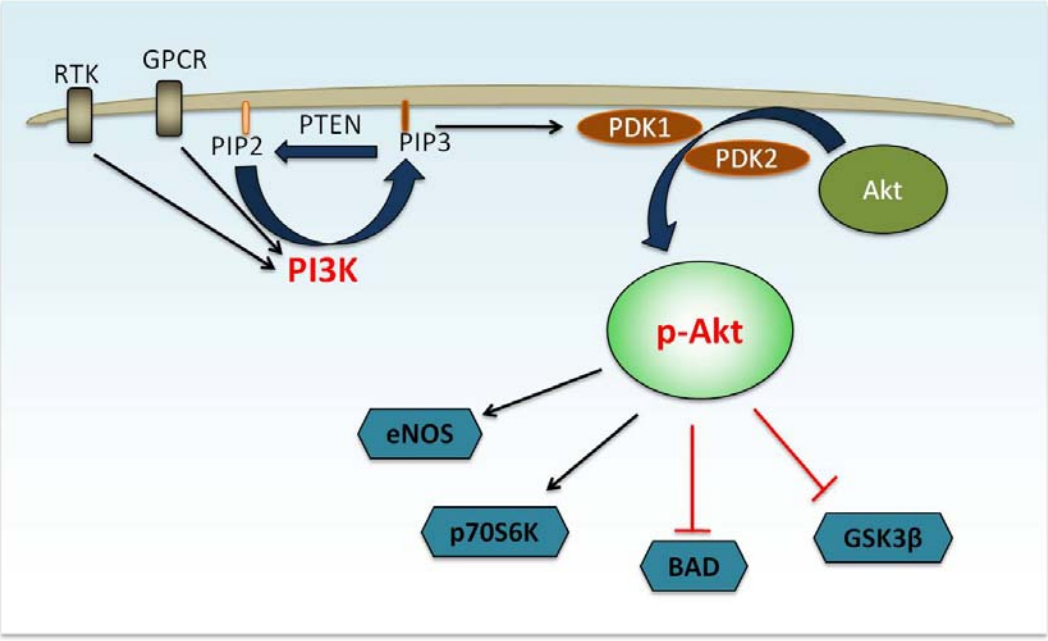

Figure 1.4 PI3K-Akt pathway. Activated PI3K phosphorylates PIP2 to PIP3. Once formed PIP3 binds to PH-domain of Akt and PDK1 and PDK1 activates Akt by phosphorylation. Activated Akt then inhibits various proteins; such as GSK-3β and BAD and stimulates anti-apoptotic proteins; such as eNOS and p70S6K. The PI3K-Akt signaling is negatively regulated by PTEN. PTEN de-phosphorylates PIP3 to PIP2 and prevents activation of Akt. Adapted from :Vivanco & Sawyers 2002.[100]

1.4.2 Mitochondrial permeability transition pore (mPTP)

Cardioprotective signaling activated by IPC, POC and many pharmacological agents involves inhibition of mPTP.[101-103] mPTP is a multiprotein complex that spans both mitochondrial membranes. Currently, the structure of mPTP is not completely clear; nevertheless, adenine nucleotide translocator (ANT) and cyclophillin-D are commonly accepted as a part of mPTP structure.[101-102] Involvement of voltage dependent anion channel (VDAC) as a structural protein of mPTP is controversial though most of the reports suggest involvement of VDAC in regulation of mPTP.[101-102]

Under normal physiological conditions, mPTP opens briefly and rapidly closes, however, following significant cellular stress, such as I/R injury, it will remain open.[101] Transient opening of mPTP allows passage of molecules less than 1500 Da but prolonged opening, such as in case of I/R injury, results in loss of mitochondrial membrane potential, release of pro-apoptotic factors and ultimately cell death.[101, 104-105] During I/R, prolonged opening of mPTP occurs in response to conformational changes in the structural proteins of the mPTP [104-105] or interaction of several pro-apoptotic molecules from the Bcl-2 family proteins, such as Bax, BAD and Bid, with the outer mitochondrial membrane.[106] Prevention of mPTP opening during reperfusion has been demonstrated to prevent cardiomyocyte death and protect the heart

against I/R injury in various animal models.[107-110] Cyclosporine A and Sanglifehrin A (immunosuppressant agents) are known to inhibit mPTP opening and have been demonstrated to have protective effects against I/R injury.[107] Cyclosporine A and its analogues prevents binding of cyclophillin-D to ANT and thereby prevents opening of mPTP.[107] Similarly, IPC and POC have also been shown to activate kinase cascade that promote cell survival by limiting mPTP induction.[101, 108-110]

1.4.3 Glycogen synthase kinase- 3β (GSK-3β)

Glycogen synthase kinase-3 is a serine/threonine kinase known to phosphorylate and thereby inhibit glycogen synthase enzymes, the rate limiting step in glycogen synthesis.[102] GSK-3 has been demonstrated to be involved with many cell processes such as the regulation of multiple transcription factors, nuclear factor κB, endoplasmic reticulum stress, embryogenesis, cell cycle progression, cell migration and most important for I/R injury the regulation of apoptosis and cell survival.[102, 111] Two isoforms of GSK-3 has been reported: GSK-3α (51 kd) and GSK-3β (46 kd). GSK-3α and GSK-3β both are constitutively active and become inactive upon phosphorylation at Ser 21 and Ser 9 by upstream kinases, respectively.[102]

GSK-3β has been demonstrated to be involved in cardioprotective signaling while GSK-3α appears to not be involved.[102-103] Inhibition of GSK-3β results in decreased apoptosis and improved metabolism

resulting in cardioprotective effects.[112-113] A number of studies have reported that the inhibition of GSK-3β plays a major role in IPC and POC induced cardioprotection.[114-115] Receptor tyrosine kinase or certain G protein-coupled receptor activation elicits cell protection by inhibiting GSK-3β, via Akt and mTOR/p70s6k, PKC or protein kinase A (PKA) dependent pathways.[101, 103, 114, 116] Activation of upstream kinases causes phosphorylation of GSK3β (phospho-GSK3β) which then binds to ANT and suppresses ANT- cyclophillin-D interaction and increases the threshold for mPTP opening.[103] Inhibition of mPTP opening in response to GSK3β inhibition has been well established.[112] The convergence of the upstream pathways via inhibition of GSK-3β on the end effectors, the permeability transition pore complex, to limit mPTP opening is the general mechanism of cardiomyocyte protection.[103]

1.4.4 Protein kinase Cε (PKCε)

The PKC family is composed of ten serine/threonine kinases that are further divided into three subfamilies based upon homology and activation requirements: conventional (α, β and γ), novel (δ, ε, η and θ) and atypical (ι and ζ).[117-118] Conventional PKC isoenzymes require Ca^{+2} and diacylglycerol while the novel subfamily of PKC isoenzymes requires diacylglycerol but not Ca^{+2} for their activation.[118] Among the novel PKC isoenzymes, PKCδ plays important role in mediating the injury associated with the reperfusion of ischemic myocardium while PKCε has

been demonstrated to play important role in mediating the cardioprotective signaling.[117-118]

The involvement of protein kinase C (PKC) in protecting heart against I/R injury was first demonstrated by Ytrehus et al who showed that inhibition of PKC resulted in loss of cardioprotection induced by IPC.[119] Further evidence has revealed several PKC isoforms play roles in protecting the heart against I/R injury; however, the epsilon isoform of PKC (PKCε) is considered as one of the critical mediators of cardioprotective signaling.[120] For example, the PKCε-selective agonist octapeptide, ΨεRACK, has been shown to protect the mouse hearts against ischemic reperfusion injury.[121] PKCε has also been reported to be involved in mediating the cardioprotective signaling activated by IPC in isolated rat heart experiments.[122] Cardiac-specific over-expression of PKCε also protects the heart against I/R injury.[123] Moreover, targeted disruption of the PKCε gene abolishes the infarct size reduction following IPC in mouse hearts.[124] Together these studies suggest that PKCε is required to induce cardioprotection. Activation of PKCε takes place in response to activation of GPCR through various molecules including adenosine, catecholamines, angiotensin II, bradykinin, and endothelin.[120] Stimulation of GPCR causes activation of phospholipase that results in formation of inositol triphosphate and diacylglycerol which activates PKC. In response to cardioprotective stimuli, phosphorylation of PKCε at serine 729 induces translocation to specific subcellular targets,

such as extracellular signal-regulated kinases (p42/p44-MAPK), a member of src family of kinases (lymphocyte-specific protein tyrosine kinase; Lck), mitochondrial ATP-sensitive K^+-channel (mitoK_{ATP} channel) and mPTP.[120] Translocation of PKCε- p42/44MAPK complex to mitochondria causes phosphorylation and inhibition of pro-apoptotic proteins such as BAD and protects the heart against I/R injury.[125] Moreover, activation of mitoK_{ATP} and inhibition of mPTP opening by PKCε also exert cardioprotective effects following I/R.[103, 126]

1.4.5 Natriuretic peptides

Natriuretic peptides are the family of hormones or paracrine factors that are involved in the regulation of cardiovascular homeostasis, fat metabolism and bone growth.[127-128] Atrial natriuretic peptides (ANP), Brain or B-type natriuretic peptides (BNP), and C-type natriuretic peptides (CNP) are three main natriuretic peptides. Other members of the natriuretic peptide family includes Dendroaspis natriuretic peptides (DNP), urodilatin, guanylin and uroguanylin[127-128]. Most known effects of ANP, BNP and CNP on the cardiovascular system are cardioprotective, natriuretic, diuretic and vasorelaxent effects.[129]

Effects of natriuretic peptides are mediated through natriuretic peptide receptors (NPR), in which three types, NPR-A, NPR-B and NPR-C, have been identified.[128, 130-131] NPR-A and NPR-B are membrane associated guanylyl cyclase receptors having 44 percent homology in their

structure, while NPR-C is the clearance receptors.[128, 130-131] Natriuretic peptide receptors are expressed on cardiomyocytes, endocardial endothelial cells and coronary vascular smooth muscle cells.[132] NPR-A is selective towards ANP compared to BNP and has least affinity for CNP. NPR-B has highest affinity for CNP and much lower affinity for ANP and BNP. NPR-C has equal affinity for ANP, BNP and CNP. [131, 133]

B-type or brain natriuretic peptide:

BNP was initially discovered in the porcine brain and therefore originally called brain natriuretic peptide.[134] BNP was later found to be most abundant in the ventricle, where it acts in an autocrine manner as a cardiac hormone.[135] BNP is also stored in a recently identified, novel population of cardiac endocrine cells, strategically located near and around blood vessels of the mammalian heart.[127] Plasma levels of BNP are markedly elevated under the pathophysiological conditions of cardiac dysfunction, including diastolic dysfunction, congestive heart failure, pulmonary embolism, and cardiac hypertrophy.[127] BNP is an early response protein where BNP expression increases within 1 hr.[136] While up-regulation of BNP expression is a widely used clinical diagnostic marker for left ventricular hypertrophy, diastolic dysfunction and heart failure, numerous studies have demonstrated antihypertrophic effects of both exogenous and endogenous BNP in experimental settings.[137]

Cardioprotective effects of BNP are mediated by NPR-A and NPR-B while the removal of the excess amount of peptides is done by clearance receptors, NPR-C. All natriuretic receptors are well expressed in the heart.[132] While the mechanism(s) of BNP-induced cardioprotection are not completely clear, stimulation of NPR-A or –B, by BNP has been demonstrated to cause increase in production of cyclic guanosine monophosphate (cGMP) triggering downstream events.[138-140] cGMP is known to bind predominately with three different binding proteins, PKG, cyclic nucleotide phosphodiesterase (PDEs) and cyclic nucleotide-gated ion channels.[127] PKG cardioprotective signaling involves activation of downstream targets including PKC and mitoK_{ATP} channels suggesting PKC and mitoK_{ATP} may be involved in BNP-induced cardioprotection.[141] Using an isolated rat heart model, D'Souza et al showed the reduction in infarct size by BNP correlated with increased cGMP levels.[139] Moreover, reduction of the infarct size was attenuated by non-specific K_{ATP} channel blocker, glibenclamide and mitoK_{ATP} channel blocker, 5-HD (5-Hydrocydecanoate).[139] Together, BNP induced cardioprotection involves NPR-A/B, cGMP, PKG, PKCε and mitoK_{ATP} channel mediated signaling.

1.5 Arachidonic acid metabolism

Arachidonic acid (AA, $C_{20}H_{32}O_2$), a 20-carbon chain polyunsaturated fatty acid, is synthesized from linoleic acid, an N-6

polyunsaturated fatty acid, found in vegetable oil, safflower, sunflower, corn oil and poppy seed.[142] Saturase and desaturase enzymes convert linoleic acid to AA.[142] Apart from the AA synthesized from the linoleic acid, AA can be obtained directly from dietary sources such as eggs, meat and dairy products. Normally, AA is found esterified into the plasma-membrane to the SN-2 position of phospholipids such as phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) of most cells, including cardiomyocytes.[143-145] In response to stimuli, such as ischemia, AA gets released from the plasma membrane by the calcium activated phospholipase A2 (PLA2).[143, 145] In the heart and coronary blood vessels, AA is metabolized by cyclooxygenase (COX), lipoxygenase (LOX) and CYP monooxygenases to a wide array of compounds (Fig. 1.5) having variety of actions including vasodilation, vasoconstriction, enhanced myocardial injury and cardioprotection.[143, 145]

LOX are the non-heme iron-containing enzymes that dioxygenate AA to bioactive metabolites such as hydroperoxyl metabolites.[146-147] There are three major LOX isozymes, 5-, 12-, and 15-LOX, that metabolize AA to the corresponding hydroperoxy eicosatetraenoic acids (5-, 12-, 15-HPETE).[148] HPETE are unstable molecules and further reduction of these metabolites by peroxydase leads to production of corresponding hydroxyl derivatives (hydroxyeicosatetrienoic acid) (5-, 8-,

12-, 15-HETE).[146] HPETE metabolites can be metabolised further into leukotrienes and lipoxins.[145, 147]

Another well-characterized enzyme involved in the metabolism of AA is the COX or prostaglandin H_2 synthase (PGHS). COX metabolize AA to bioactive metabolites; prostaglandins (PG) and thromboxanes (TX).[149] There are two COX isozymes COX-1 and COX-2. COX-1 is constitutively expressed while COX-2 expression is inducible by various stimuli.[150] Both the COX isozymes catalyze the conversion of AA to prostaglandin G_2 and H_2 (PGG_2 and PGH_2), the first two steps in prostaglandin biosynthesis.[149-150] COX first causes oxidation of AA to produce PGG_2 and subsequent reduction of PGG_2 to the PGH_2. Further, by various enzymes and nonenzymic reactions PGH_2 gets converted into the primary prostanoids, PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and TXA_2.[149]

Apart from LOX and COX, AA can also be metabolized by a third and less characterized pathway; the CYP monooxygenase pathway.[145, 151] CYP monooxygenases metabolize AA to hydroxyicosatetraenoic acids and epoxyeicosatrienoic acids.[145, 151-153] AA is catalyzed by CYP epoxygenases (CYP2C, 2J) to 4 regioisomers of epoxyeicosatrienoic acids (5,6-, 8,9-, 11, 12- and 14,15-EETs) that subsequently get hydrolyzed to their corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH).[145, 151, 153] In addition, CYP hydroxylases (CYP4A, 4F) convert AA to several HETEs including 19- and

20-HETE.[152] A number of other metabolites of the CYP pathway have also been identified and include 19- and 20-OH PGs. In this thesis, the cardioprotective effects of CYP epoxygenase metabolites of arachidonic acids are examined.

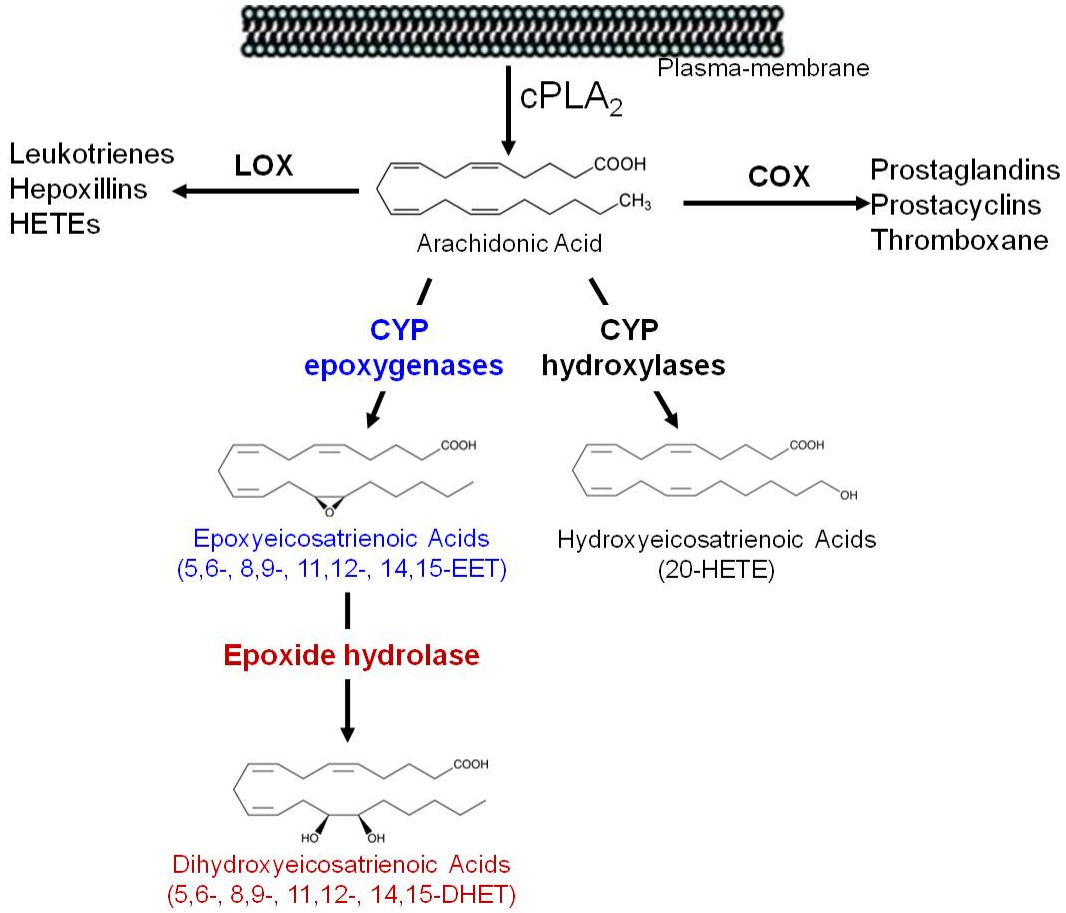

Figure 1.5 Arachidonic acid metabolism.

Free arachidonic acid can be metabolized by LOX, COX and CYP monooxygenases to a large number of biologically active compounds. CYP epoxygenases converts arachidonic acid to EETs. Further, EETs get metabolized by epoxide hydrolase to less bioactive DHETs. Adapted from: Seubert et al 2007.[154]

1.6 Cytochrome P450 (CYP)

CYP genes encode a super-family of mixed function monooxygenases which are comprised of more than 6000 individual enzymes.[155] CYP enzymes are produced by a wide variety of organisms including both prokaryotes and eukaryotes, with at least 57 different CYP genes identified in the human genome (http://drnelson.utmem.edu/human.P450.table.html).[155-156] CYPs play a major role in the metabolism of foreign lipophilic compounds, including drugs and chemical carcinogens, as well as endogenous compounds such as steroids, fat-soluble vitamins, fatty acids and biogenic amines.[151, 155-156] While multiple CYP enzymes have overlapping substrate selectivity; many individual isoforms demonstrate unique regio- or stereoselectivity toward particular substrates, for example testosterone or arachidonic acid.[151] For example, each CYP epoxygenase metabolize AA to EETs and produce several regioisomers with one form usually predominating. The rat kidney CYP2C23, catalyzed the enantioselective epoxidation of AA to 5,6-, 8,9-, 11,12-, and 14,15-EET, with 11,12-EET as its major product.[151, 157] On the other side, recombinant human CYP2J2 preferably produce 14,15-EET as compared to other three EET-isomers.[158] Epoxidation of AA by CYP2J2 at the 14,15-olefin has been reported to be highly enantioselective for (14R,15S)-EET (ratio of antipodes 3:1).[158] CYP expression and activity is under the control of hormones, growth factors, and transcription factors. Indeed, different CYP

subfamilies can display complex sex-, tissue-, and development-specific expression patterns.[159-160] In addition, CYP expression and activity can be influenced by various factors such as genetic variation, presence of inhibitor or inducer and disease state. [161-166]. Importantly, variations in expression and activity can lead to significant changes in metabolism and physiological responses, which in turn can contribute to failure of drug treatment or adverse reactions.[164-166] Considering the significant role of CYP in drug metabolism and synthesis of endogenous molecules, there remains a continued interest in understanding their role IHD.

1.6.1 CYP expression in the heart

In mammalian cells, CYP enzymes are found localized to the endoplasmic reticulum with limited expression occurring in mitochondria.[167] While CYPs are predominantly expressed in the liver, there are significant levels of CYP isozymes found in extrahepatic tissues such as lung, kidney, gastrointestinal tract and heart.[159-160, 168] When compared to the liver, the metabolic capacity and drug clearance of these organs is low. However, extra-hepatic CYP can contribute significantly to endogenous tissue function, drug biotransformation and systemic exposure to foreign compounds.[159] Tissue-specific differences in CYP-mediated metabolism of certain chemicals are the result of different expression and/or regulation of the relevant P450 enzymes. Interest in the regulation of CYP enzymes in extrahepatic tissues is important from a

detoxication as well as a bioactivation perspective. A compound readily metabolized in the liver may be toxic in some other tissue due to differences in CYP expression. CYP expression is influenced by both endogenous and exogenous factors ranging from environmental pollutants to hormones. Emerging evidence suggests a role for CYP enzymes in the cardiovascular system; however, specific understanding about CYP isozyme regulation and function within heart is limited.

Cardiac expression of CYP subfamilies indentified in mammalian species include, CYP1A, CYP1B, CYP2A, CYP2B, CYP2D, CYP2E, CYP2J, CYP2R, CYP2S, CYP2U, CYP4A, CYP4B, CYP4F and CYP11B.[155, 159-160, 168-171] In healthy human heart, CYP1A2 mRNA is absent while CYP1A1 is expressed in very low amounts.[155] Data suggests that CYP1A1 mRNA is expressed only in the left ventricle in healthy human heart while in patients with dilated cardiomyopathy (DCM) it can be detected in the right ventricle, ascending aorta, pulmonary aorta and right atrium.[170] Comparatively, in rat heart, CYP1A1 is not constitutively present in the cardiovascular system but can be induced.[170-172] CYP1B1 mRNA expression was observed in normal human heart in relatively high amount and protein was detected in the left ventricle of the normal rat heart.[155, 170]

Cardiac expression of CYP2 isozymes responsible for epoxygenase activity, CYP2C8, CYP2C9 and CYP2J2 are reported in the

literature. Bieche et al. suggested these isoforms are constitutively expressed in the normal heart and Delozier et al. reported induced expression of CYP2C8 and CYP2C9 mRNA and protein following ischemic injury.[155, 163] In contrast, significant levels of CYP2J2 expression were observed in normal hearts with levels 1000 times higher than CYP2C8 and 2C9.[163] There is limited data on the cardiac expression of other CYP2 sub-families. CYP2B6/7 has been reported in right ventricle and aorta of the patient with DCM.[173] CYP2D6 and CYP2E1 mRNA were observed in normal hearts as well as individuals with DCM.[155, 173] In patients with DCM, CYP2D6 was only expressed in right ventricle. In these patients CYP2E1 was expressed in both atria and ventricles but it was absent in aorta and pulmonary artery.[173]

Real time-PCR analysis of heart tissue revealed no expression of CYP3A4, 3A5, 3A7 and 3A47 mRNA.[155, 173] In contrast to the mRNA expression, Minamiyama et al. has reported intense binding of CYP3A4 in immunohistochemistry of heart tissues.[174] In CYP4 subfamily, CYP4A1, CYP4A2 and CYP4F activities were detected in dog and rat heart tissue while CYP4F12 mRNA was detected in human heart.[170] CYP4B1 mRNA was detected only in the right ventricle of the patients with DCM.[173] No consistent results were observed in the patients with DCM in CYP8-19 expression.[173] CYP11A mRNA can be observed in normal and failing heart while CYP11B mRNA is only expressed in the failing heart. [170]

1.6.2 CYP and Cardiovascular diseases

CYP epoxygenases and CVD

CYP epoxygenases biosynthesize four regioisomeric cis-epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) from AA, all of which are biologically active.[175] CYP epoxygenases mainly include CYP1A, CYP2B, CYP2C, CYP2E, and CYP2J subfamilies.[176] However, in the cardiovascular system, CYP2C and CYP2J subfamilies are considered as the major enzymes involved in EETs synthesis.[177] Numerous studies have explored the effects of EETs in cardiovascular system and demonstrated beneficial effects. EETs produce vasodilatation [178-180], inhibit vascular inflammation [181-182], inhibit platelet aggregation [183], inhibit apoptosis [184-186] and protect the heart against I/R injury.[187] Cardioprotective effects of EETs are discussed in detail in section 1.8.

CYP2C, Reactive Oxygen Species (ROS) and CVD

In contrast to beneficial effects reported for CYP epoxygenases within the CVS, evidence indicates that adverse effects are associated with CYP2C.[188-191] CYP2C9 mediated reactions produce ROS that have detrimental effects.[188-189, 192-194] Edin et al. reported decreased postischemic functional recovery in the hearts isolated from mice with endothelial expression of human CYP2C8.[195] The decreased postischemic function was attributed to increased production of ROS and

linoleic acid metabolites (DiHOMEs).[195] Indeed, ROS can induce apoptotic responses, mitochondrial damage and worsen ischemic reperfusion injury.[192-193] CYP2C9-mediated generation of ROS in arteries reacts with NO to produce $ONOO^-$, thereby decreasing NO bioavailability that leads to vasoconstriction and vascular dysfunction.[188] Inhibition of CYP2C9 by sulphaphenazole, a specific CYP2C isozyme inhibitor, has been shown to increase forearm blood flow in patients with coronary artery disease.[194] However, CYP2C9 inhibitors do not alter the forearm blood flow induced by acetylcholine and sodium nitroprusside in healthy individuals.[196] These studies suggest that decreased bioavailability of NO due to ROS generation by CYP2C9 can contribute to vascular dysfunction in CAD patients. [194] Recently, Hunter et al. demonstrated that pretreatment with sulphaphenazole prevented cardiac allograft vasculopathy in a rat heterotopic heart transplant model.[197] The results further suggested that inhibition of CYP2C during peritransplant reduces oxidative damage and increases NO bioavailability. This lead to inhibition of smooth muscle cell proliferation and intimal hyperplasia which reduced endothelial dysfunction associated with cardiac allograft vasculopathy.[197] Interestingly, Gross et al. reported attenuation of cardioprotective effects of EETs by free radical scavenger, 2-mercaptopropionyl glycine, which suggests that initial burst of ROS is important for EET mediated cardioprotection.[198] Together, these studies

highlight the complexity of the CYP epoxygenases and contrast the effects they have on the cardiovascular system.

CYP ω-hydroxylases and CVD

CYP ω-hydroxylases, such as CYP4A and 4F, which biosynthesize hydroxyeicosatetrienoic acids (17-, 18-, 19-, and 20-HETE) are present in heart. HETEs act as potent vasoconstrictors by inhibiting large conductance Ca^{2+}-activated K^+ channels (BK_{Ca} channels), which depolarizes vascular smooth muscle membrane. Induction of CYP ω-hydroxylase, CYP4A family enzymes, has been reported in liver and kidney of diabetic rats.[199] Recently, Yousif et al. demonstrated significant decreases in cardiac functional recovery in diabetic rats compared to normal rats following I/R injury.[200] CYP ω-hydroxylase activity was two times higher in diabetic rat hearts compared to normal hearts.[200] Inhibition of CYP ω-hydroxylase and therefore decreased formation of 20-HETE reduces the damage to the heart following I/R.[200-202] These results suggest that increased activity of CYP ω-hydroxylase and enhanced formation of 20-HETE contributes to exaggeration of I/R injury. There was also a non-significant trend towards an increase in the CYP epoxygenase activity in diabetic rats but this may be the effect of activation of the protective signaling pathways in response to increased 20-HETE levels in the heart.[200] In another study, Aboutabl et al. demonstrated increased expression of CYP- ω-hydroxylase and 20-

HETE:EETs ratio in the heart treated with 3-methylcholanthrene and benzo(a)pyrene.[203] Moreover, the study reported that the increased CYP- ω-hydroxylase contributed toward development of cardiac hypertrophy.[203] Together, these reports suggest that differences in the expression and/or activity of specific CYP epoxygenase and hydroxylase enzymes can alter the delicate balance between EETs and HETEs which can then lead do adverse outcomes.

1.7 Clinical relevance of EETs in cardiovascular disease

CYP genes exhibit genetic variability which can significantly influence an individual's response to therapy and disease.[204] However, only some CYP isoforms involved in drug metabolism have been reported to have functionally relevant polymorphisms, including CYPs 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4/5.[205] Importantly, CYPs are involved in metabolism of exogenous and endogenous substrates; therefore genetic polymorphisms not only influence drug treatment but have been associated with the development of cardiovascular disease.[206] Polymorphisms in the CYP epoxygenases and sEH, have been associated with CVD risk in humans (extensive review by Theken and Lee [207] and Zordoky and El-Kadi [177]).

Several polymorphisms have been identified in CYP2J2. A common functionally relevant variant found in the proximal promoter region results

in disruption of the Sp1 transcription binding site (G-50T) interfering with transcription and reducing expression.[161, 208] Speicker et al. provided the first evidence demonstrating a correlation between this G-50T polymorphism in *CYP2J2* gene and coronary heart disease in a German population.[161] Moreover, the G-50T mutation resulted significantly lower plasma concentrations of stable EET metabolites in the individuals with the G-50T SNP.[161] Similarly, Liu et al. reported significant association between G-50T polymorphism in CYP2J2 and increased risk of premature MI.[209] The risk of premature MI increased significantly in the smoking-patients carrying T allele compared to non-smokers. Consistent with increased risk of premature MI, patients carrying T allele had lower EET-metabolite levels in plasma compared to GG patients. EET-metabolite level further decreased in the smoking-patients carrying T allele.[209] However, Lee et al. reported lower incidents of CHD in an African-American population carrying G-50T variation in atherosclerosis risk in community (ARIC) study.[210] Haffmann et al. did not find any significant association between CYP2J2 -50G>T polymorphism and CAD in the Ludwigshafen Risk and Cardiovascular Health (LURIC) cohort.[162] On the other side, evidence for an association between CYP2J2 polymorphisms found in the intronic regions and increased risk of myocardial infarction was reported in a case-control study in Washington State.[211] For CYP2C, a recent study reported that polymorphisms in CYP2C8 and 2C9 have no correlation with myocardial ischemia or

ischemic stroke.[211] However, CYP2C8 genetic variations have been shown to be associated with higher risk of coronary heart disease in smokers.[210] These studies demonstrate that decreased synthesis of EETs due to polymorphism in CYP epoxygenases is associated with increased risk of IHD.

The *EPHX2* gene encodes for sEH and there are 44 known single nucleotide polymorphisms (SNPs) and 1 insertion/deletion of the human *EPHX2* gene.[212] Various studies have demonstrated that the *EPHX2* gene variant is a risk factor for CVD. Individuals carrying the K55R genetic variation in *EPHX2* have higher sEH activity and this genetic variation has been demonstrated to be significantly associated with the risk of IHD in Caucasians in ARIC study.[213] Another study reported that the polymorphism in *EPHX2* gene is associated with subclinical CVD. The study, involving 982 European-American and 176 African- American subjects, reported that the R287Q polymorphism was associated with coronary artery plaques in European Americans.[214] Furthermore, in African-Americans, R287Q polymorphism was demonstrated to be associated with significantly greater risk for coronary artery calcified plaque.[215] Similarly, in Whites, a polymorphism in Intron 11 of the *EPHX2* gene was associated with significantly greater risk for coronary artery calcified plaque.[215] These studies demonstrate that decreased plasma-levels of EETs either by increased metabolism of EETs by increased sEH or decreased synthesis of EETs by CYP epoxygenases

are associated with higher risk of IHD. Together, despite some variability, research to date demonstrates that EETs can be the endogenous molecule that protects the heart against IHD.

1.8 Epoxyeicosatrienoic acid and cardioprotection

EETs are important components of many intracellular signaling pathways involved in vasodilatory, anti-inflammatory and cardioprotective responses in the cardiovascular system.[143, 154, 216] Moreover, EETs are also demonstrated to affect various cardiac ion channels through direct and indirect mechanisms.[187, 217] Xiao et al. suggested that EETs activate cAMP-protein kinase A-dependent phosphorylation of the L-type Ca^{2+} channel and enhance I_{Ca} in transgenic mice overexpressing human CYP2J2.[217] Lu et al. demonstrated that 11,12-EET could increase the activity of ATP-sensitive K^+ channels by directly reducing sensitivity to ATP.[218] EETs are known to hyperpolarize and relax vascular smooth muscle cells by activating calcium-sensitive potassium (BK_{Ca}) channels as well act as potent vasodilators in the coronary microcirculation.[219-220] Thus, alteration in CYP epoxygenases may affect steady-state cellular levels of these bioactive eicosanoids in vivo and could potentially influence cardiac function.

Moffat et al first reported the effects of the four isomers of EETs on cardiovascular function under normal condition as well as low-flow

ischemia in isolated guinea pig hearts and isolated ventricular myocytes.[221] No effect of EETs was observed regarding heart rate, coronary perfusion pressure, or energy metabolite content after 60 min low-flow ischemia and 30 min of reperfusion.[221] While 5,6- and 11,12-EET significantly increased cell shortening as well as intracellular calcium concentrations, 8,9- or 14,15-EET was without effect.[221] Opposite to the first report presented by Moffat et al, most of the subsequent studies reported protective effects of EETs against I/R injury.[40, 168, 187, 198, 222-224] Wu et al cloned both human and rat isoforms CYP epoxygenase in the heart, CYP2J2 and CYP2J3 respectively.[158, 168] Following the identification of CYP epoxygenases in the heart, protective effect of EETs was reported in isolated rat hearts against global no-flow ischemia. [168] Cardiomyocyte specific overexpression of CYP2J2 (CYP2J2 Tr) have been demonstrated to improve postischemic functional recovery in isolated mouse heart perfusions.[187] The effects were mediated through mitoK_{ATP} and MAPK 42/44.[187] Our group demonstrated protective effects of EETs in a global knock out mouse model for sEH (sEH null or sEH KO).[222] Lack of sEH expression in these mice resulted in increased epoxide:diol ratio that correlated with improved postischemic function in these mice compared to littermate controls.[222] For the first time this study reported involvement of PI3K in EET-induced cardioprotection.[222] In addition, recent data show reduced electrophysiological abnormalities following I/R, in CYP2J2 transgenic mice or wild-type mice treated with

exogenous EETs and these effects of EETs are mediated through K_{ATP} channels and PKA.[225] Similarly, the inhibition of sEH enzyme either by pharmacological agents such as CDU, AUDA and AUD-BE, results in elevated cellular EET levels producing significant cardioprotective actions.[226]

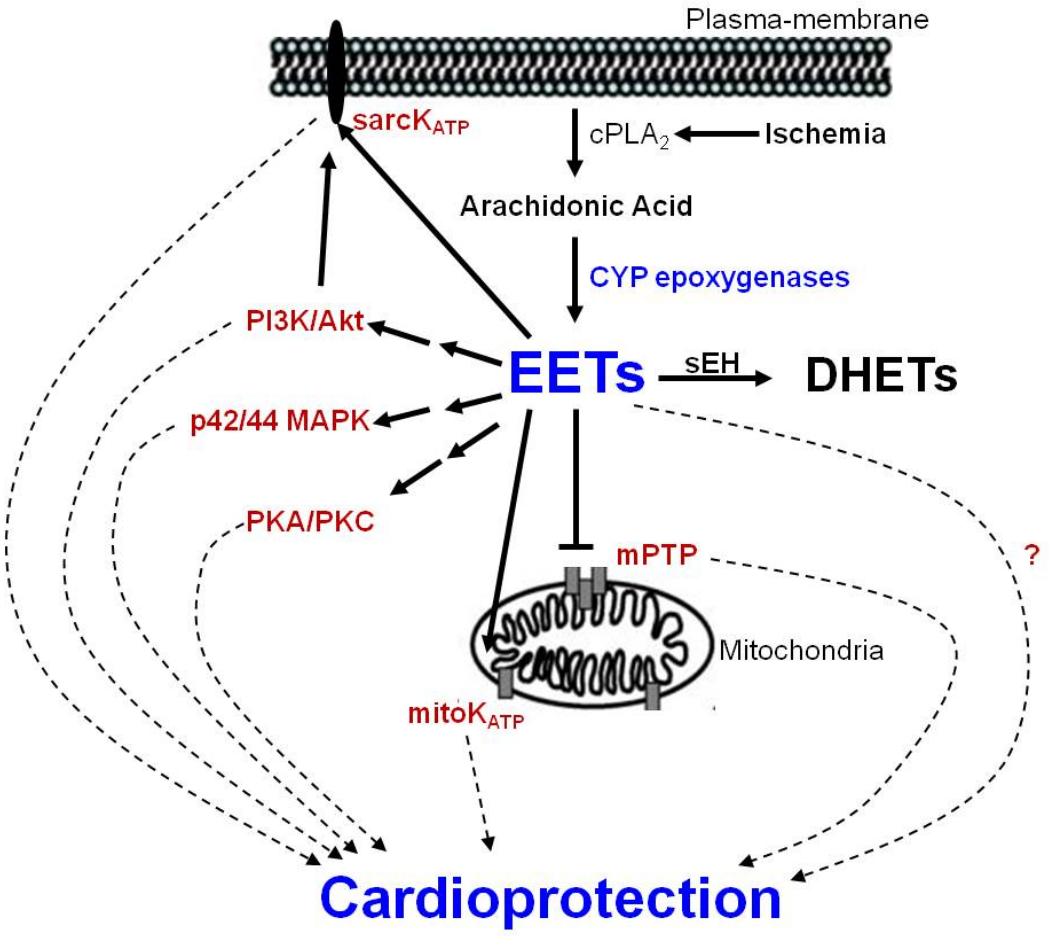

Figure 1.6 Mechanism of cardioprotection induced by EETs.

In response to ischemia $cPLA_2$ gets activated that releases AA from plasma membrane. Oxidation of the free AA by CYP epoxygenases leads to increase in intracellular levels of EETs. These EETs can act on various intracellular targets including p42/44 MAPK, K_{ATP} channels ($sarcK_{ATP}$ and $mitoK_{ATP}$), PKA and PKC and induce cardioprotection. Adapted from: Seubert et al 2007.[154]

1.9 Thesis overview

1.9.1 Rationale

As discussed earlier, over the past two decades a range of studies have demonstrated that EETs act as critical intracellular mediators that can reduce the infarct size, initiate protective responses following I/R injury and improve postischemic left ventricular function. Previous studies from other laboratories and our group demonstrated involvement of PI3K-Akt pathway, p42/44 MAPK and K_{ATP} channels in EET-induced cardioprotection. Moreover, recent studies demonstrated that EET-induced cardioprotection converges onto mitochondria, preventing damage that results from I/R injury. Despite efforts for exploring EET-induced cardioprotection, the exact mechanism(s) through which EETs render protection is still not clear. Our preliminary data suggests that EETs induce BNP mRNA expression following I/R and therefore BNP may mediate EET-induced cardioprotective signaling. Moreover, the cardioprotective effects of EETs have been reported in young animal models and there is lack of information about EET-induced cardioprotection in aged animal models. The overall aim of the current study is to investigate the mechanism(s) of EET-mediated cardioprotection and study the effectiveness of EETs in aged animal models.

1.9.2 Hypothesis

1. Increased levels of EETs by inhibition of sEH protect the heart against I/R injury.
2. The cardioprotective effects of EETs are mediated, at-least in-part, through BNP and PI3K pathways.
3. EETs maintain postischemic cardiac function in young as well as aged animals.

1.9.3 Thesis aims

1. To investigate the effects of the pharmacological inhibitor of sEH on postischemic cardiac function. (Described in Chapter 2)
2. To study the role of BNP in EET-induced improved postischemic cardiac function. (Described in Chapter 3)
3. To investigate the effect of aging on EET-induced protection against I/R injury. (Described in Chapter 4)

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Chapter 2

Inhibition of soluble epoxide hydrolase by *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) is protective against ischemia reperfusion injury

[2]A version of this chapter has been published: Chaudhary KR, Abukhashim M, Hwang SH, Hammock BD, Seubert JM. Inhibition of soluble epoxide hydrolase by trans-4- [4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid is protective against ischemia-reperfusion injury. *J Cardiovasc Pharmacol. 2010 Jan;55(1):67-73*.

2.1 Introduction

Arachidonic acid, is a polyunsaturated fatty acid that is present in the phospholipids of cell membranes which can be released into cytosol in response to stressors such as ischemia.[1-2] Released free AA can then be metabolized by cyclooxygenases, lipoxygenases, and cytochrome P450 (CYP) monooxygenases.[1, 3] Biologically active regioisomers, epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) are products by CYP monooxygenases.[2-3] EETs can be reincorporated into phospholipid membranes or metabolized to smaller reactive epoxides by β-oxidation.[4-5] However, the predominant pathway of EET metabolism is conversion to the less active vicinal diols, dihydroxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-DHET), by soluble epoxide hydrolases (sEH).[4-5]

EETs act as important cellular lipid mediators in the cardiovascular, renal and nervous systems.[3, 6-8] EETs have been shown to have protective effects against ischemia reperfusion injury.[1, 9-14] Our current understanding of the cardioprotective mechanism(s) of EETs suggest involvement of signaling pathways including phosphoinositide 3-kinase (PI3K) – Akt, increased secretion of cardiac hormones, and activation of cardiac ion channels such as ATP-sensitive K^+ channels.[1, 9-10, 12-13] Recent evidence indicates that PI3K/Akt or natriuretic peptide pathways activated by EETs converge onto the mitochondria thereby limiting mitochondrial damage from ischemia reperfusion injury.[10, 12]

Previously we demonstrated that targeted deletion of sEH gene is protective against ischemia reperfusion injury.[1, 10] Recently, Motoki et al. has reported pharmacological inhibition of sEH with 12-(3-adamantan-1-yl-ureido)-dodecanoic acid *n*-butyl ester (AUDA-nBE) was cardioprotective.[15] In the present study we report cardioprotective effects of more potent, water soluble and metabolically stable sEH inhibitor (sEHi), *trans*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) than AUDA-nBE. Moreover, our data demonstrate marked reduction in infarction and improved contractile function at nanomolar concentrations which involved the PI3K pathway.

2.2 Material and Method

2.2.1 Animals

All experiments used male and female mice aged 3-5 months, weighing 22-33 g and were treated in accordance with the guidelines of Health Science Laboratory Animal Services (HSLAS), University of Alberta. C57BL6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ). A colony of mice with targeted disruption of the *Ephx2* gene (sEH null) and backcrossed onto a C57BL6 genetic background for more than 7 additional generations, is maintained at the University of Alberta.

2.2.2 Chemicals

EETs were a kind gift from Dr. J. R. Falck (University of Texas Southwestern Medical Center, Dallas, TX). Stock solutions (5 mM) were dissolved in 100% ethanol and working solutions were diluted in perfusion buffer (1 μM). The sEHi, *trans*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB), was synthesized in the laboratory of Dr. Bruce Hammock (UC Davis) and dissolved in DMSO to make 10 mM stock solution. The putative EET receptor antagonist, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) was a kind gift from Dr. J. R. Falck and dissolved in 100% ethanol to make 5 mM stock solution. PI3K inhibitors, Wortmannin (Sigma-Aldrich, Oakville, ON) and LY294002 (Cell Signaling Technology, Inc., Danvers, MA), were dissolved in DMSO to make 5 mM and 50 mM stock solutions, respectively.

2.2.3 Isolated heart perfusions

Hearts were perfused in the Langendorff mode as previously published.[1, 10] Briefly, hearts from age/sex-matched mice were perfused in a retrograde fashion at constant pressure (90 cmH_2O) with continuously aerated (95%O_2/5%CO_2) Krebs-Henseleit buffer at 37˚C. Hearts were perfused with buffer for 40 min of stabilization period and then subjected to 30 min global no-flow ischemia, followed by 40 min reperfusion. To determine *t*-AUCB dose-response, hearts from C57Bl6 mice were perfused with increasing concentrations of *t*-AUCB (0, 0.05, 0.1, 0.5 or 1μM). For some experiments, hearts were perfused for 40 min baseline, subjected to 30 min ischemia and then perfused with 0.1 μM *t*-AUCB in the presence or absence of putative EET receptor antagonist 14,15-EEZE (10 μM), PI3K inhibitors wortmannin (200 nM) or LY294002 (5 μM). The percentage of left ventricular developed pressure (%LVDP) at 40 min of reperfusion (R40), as compared to baseline LVDP, was taken as a marker for recovery of contractile function. After 40 min of reperfusion, hearts were immediately frozen and stored below -20˚C.

2.2.4 Infarct size analysis

To determine the amount of infarction, following 40 min of stabilization period and 30 min global no-flow ischemia, hearts were reperfused for 2 h. After 2 h reperfusion, hearts were perfused with 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in Krebs-Henseleit buffer at 37 °C for 10 min, then fixed in formalin and cut into thin

cross-sectional slices. The area of infarction was quantified by measuring stained (red, live tissue) and unstained (white, necrotic) regions using Image J (NIH, USA). Infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas from all sections by the sum of LV areas from all sections and multiplying by 100.

2.2.5 Statistical analysis

Values expressed as mean ± standard error of mean (SEM). Statistical significance was determined by the unpaired Student's *t*-test and one-way ANOVA followed by Newman-Keuls and Duncan's tests to assess differences between groups. Values were considered significant if $p < 0.05$.

2.3 Results

2.3.1 Cardioprotective effects of *t*-AUCB

Recent evidence suggests that sEH is a good target in the prevention of ischemia reperfusion injury.[1, 10, 15] To examine the effects of pharmacological inhibition of sEH on ischemia reperfusion injury, we first performed a dose- response study. We perfused the WT mouse hearts with 0, 0.05, 0.1, 0.5 and 1 μM of *t*-AUCB and monitored LVDP for postischemic functional recovery. No significant differences in baseline contractile function were observed between the groups, except LVDP in 1 μM *t*-AUCB treated hearts, during aerobic baseline perfusion (Table 2.1). Hearts perfused with *t*-AUCB had significantly improved postischemic recovery of LVDP compared to control mice (Fig 2.1 and Table 2.1). The most improved functional recovery occurring at the mid-dose (0.1 μM) (Fig 2.1). Therefore, 0.1 μM *t*-AUCB was used for further study the cardioprotective effects.

To further assess the cardioprotective effects of *t*-AUCB, we analyzed the infarct size following 2 h reperfusion in *t*-AUCB treated hearts and vehicle control hearts. Significant decrease in infarct size was observed in the hearts treated with 0.1 μM *t*-AUCB compared to vehicle controls (Fig 2.2). Consistent with 40 min reperfusion, postischemic functional recovery remained significantly higher at 2 h reperfusion in *t*-AUCB treated hearts compared to vehicle controls (LVDP= 43.3±13.3% vs. 21.2±1.8%).

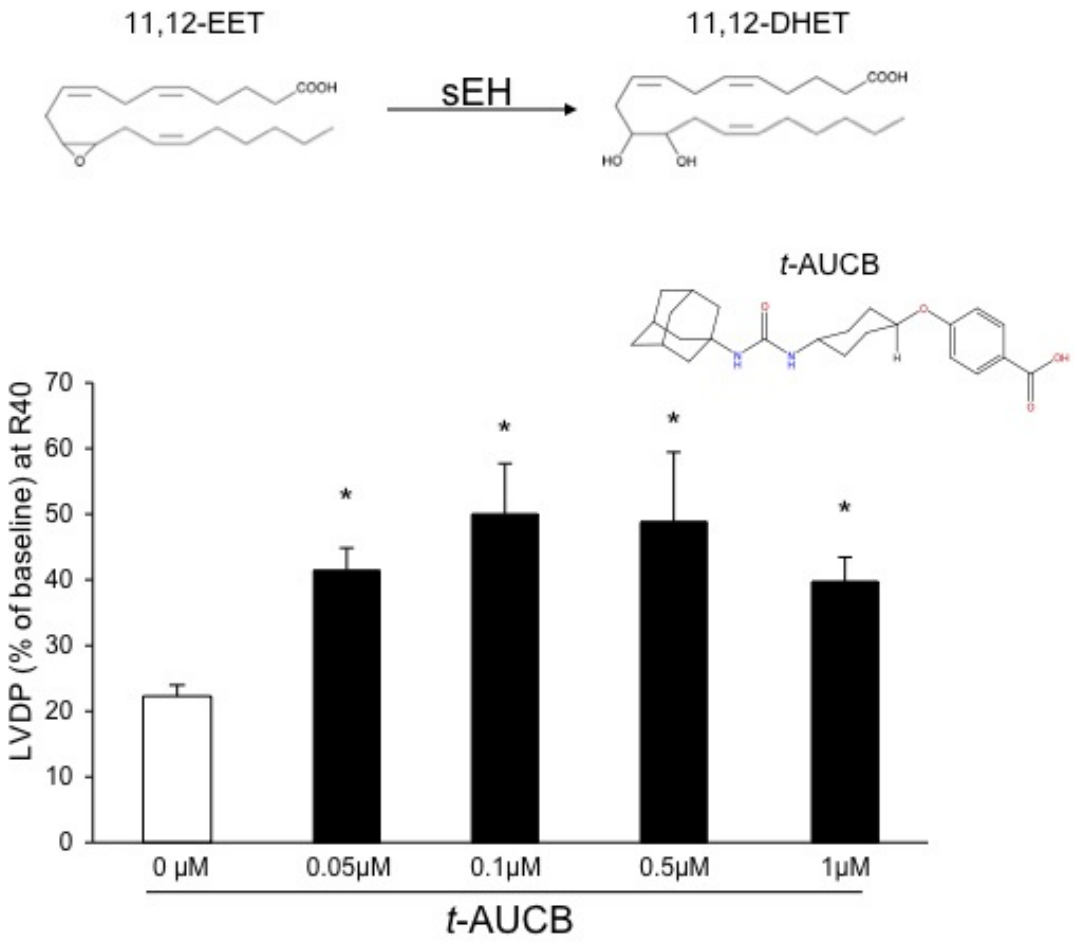

Figure 2.1 sEHi and post-ischemic functional recovery. LVDP recovery at 40min of reperfusion in WT hearts treated with *t*-AUCB (0, 0.05, 0.1, 0.5 or 1μM). Values represent mean±SEM; n=4-11 per group; *, $p<0.05$ vs. control.

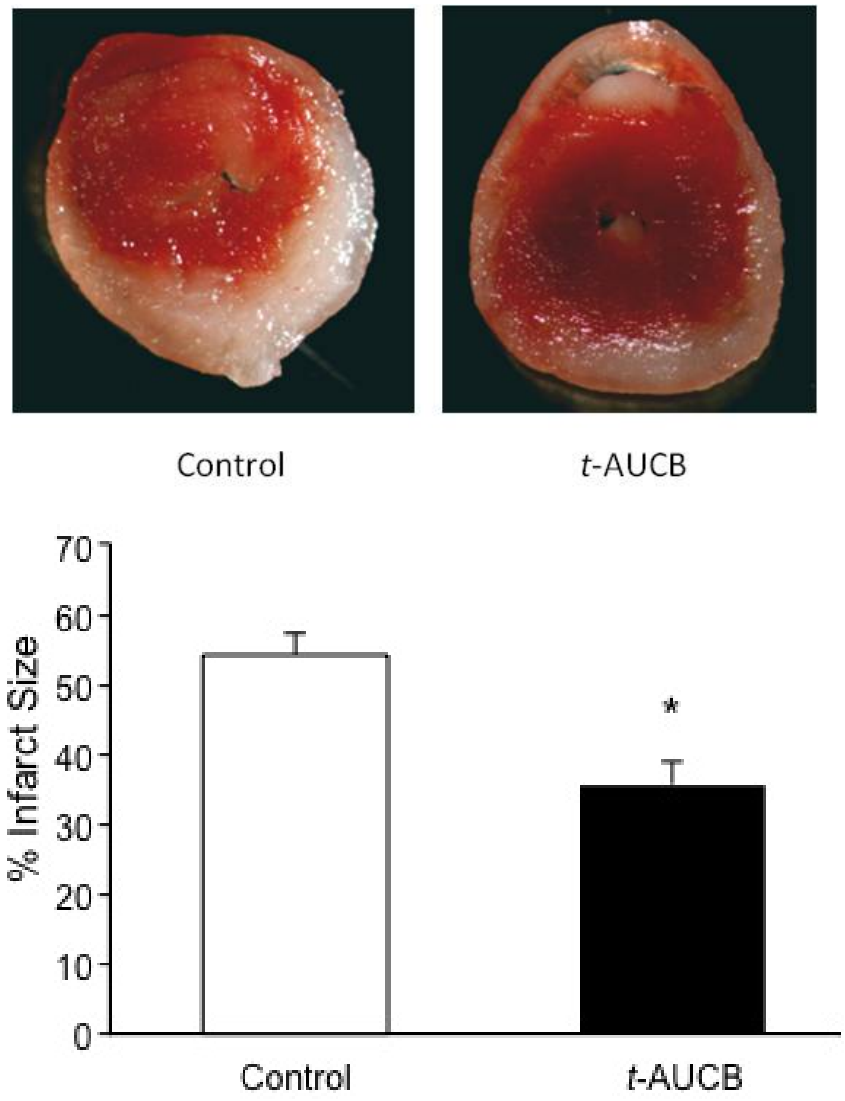

Figure 2.2 Infarct size analysis. Representative images of TTC staining and quantification of infarct size in *t*-AUCB and vehicle control hearts. Surviving tissue stained red with TTC and infracted white tissue. Values represent mean±SEM; n=4; *, $p<0.05$ vs. control.

Table 2.1 Cardiac parameters for *t*-AUCB dose response

	Vehicle control (n=11)	0.05 μM *t*-AUCB (*n*=4)	0.1 μM *t*-AUCB (*n*=6)	0.5 μM *t*-AUCB (n=5)	1 μM *t*-AUCB (n=4)
Isolated Perfused Heart - Preischemic					
LVDP (cmH_2O) (Baseline)	112.7±7.9	126.9±13.2	103.9±5.7	132.4±16.1	168.4±12.5*
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (Baseline)	3249±294	5021±318	3223±213	3757±517	4089±451
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (Baseline)	-2683±188	-3926±225	-2670±168	-3054±398	-3688±333
HR, perfused (beats/min) (Baseline)	291±19	334±13	355±19	277±46	268±21
Isolated Perfused Heart - Postischemic					
LVDP (cmH_2O) (R40)	25.1±2.9	52.0±5.6*	47.0±8.8*	57.2±14.7*	51.8±9.4*
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (R40)	807±92	1582±115*	1404±289*	1697±415*	1477±275*
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (R40)	-688±82	-1370±143*	-1326±169*	-1367±320*	-1285±203*
HR, perfused (beats/min) (R40)	323±16	337±25	340±16	347±21	298±27

Hemodynamic parameters were measured in isolated-perfused hearts. Values represent mean±SEM, * $p<0.05$ vs vehicle control. LVDP, left ventricular pressure, HR, heart rate.

2.3.2 Pharmcological or genetic inhibition of sEH and cardioprotection

To compare the cardioprotective effects of genetic and pharmacological inhibition of sEH, we perfused WT mice with 0.1 μM *t*-AUCB and sEH null mice with vehicle. Significant increase in postischemic functional recovery of sEH null (LVDP= 47.3±6.2%) and *t*-AUCB perfused WT (LVDP= 48.0±7.2%) was observed following ischemia reperfusion protocol, compared to vehicle controls (LVDP= 22.4±1.7%) and WT (LVDP= 19.7±3.8%) (Fig 2.3, Table 2.2). No significant differences were observed in cardiac parameters during baseline (Table 2.2). Consistent with LVDP, rate of contraction and relaxation were significantly higher in sEH null hearts and *t*-AUCB treated hearts following ischemia reperfusion (Table 2.2). No significant differences were observed in heart rate during baseline or following ischemia reperfusion (Table 2.2). The improved function was evident within 30 min of reperfusion following treatment with 0.1 μM *t*-AUCB and sEH null mice, which persisted throughout the recovery period (Fig 2.4).

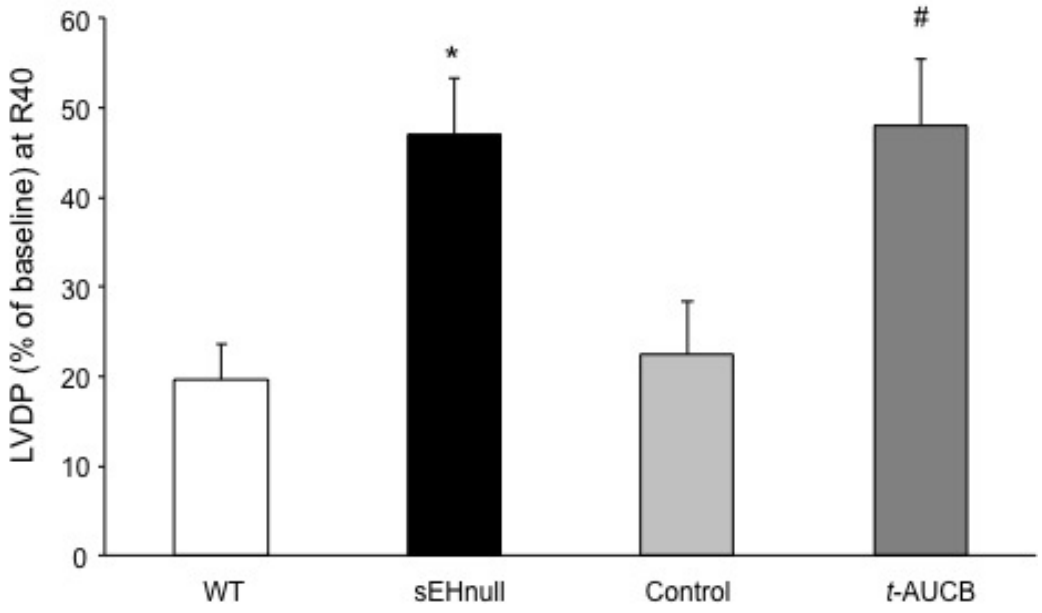

Figure 2.3 Effect of genetic or pharmacological inhibition of sEH LVDP recovery LVDP recovery at 40min of reperfusion in WT hearts, sEH null, vehicle control and WT treated with *t*-AUCB (0.1μM). Values represent mean ± SEM; n=5-11 per group; *, $p<0.05$ vs. WT; $^{\#}$, $p<0.05$ vs. Vehicle control.

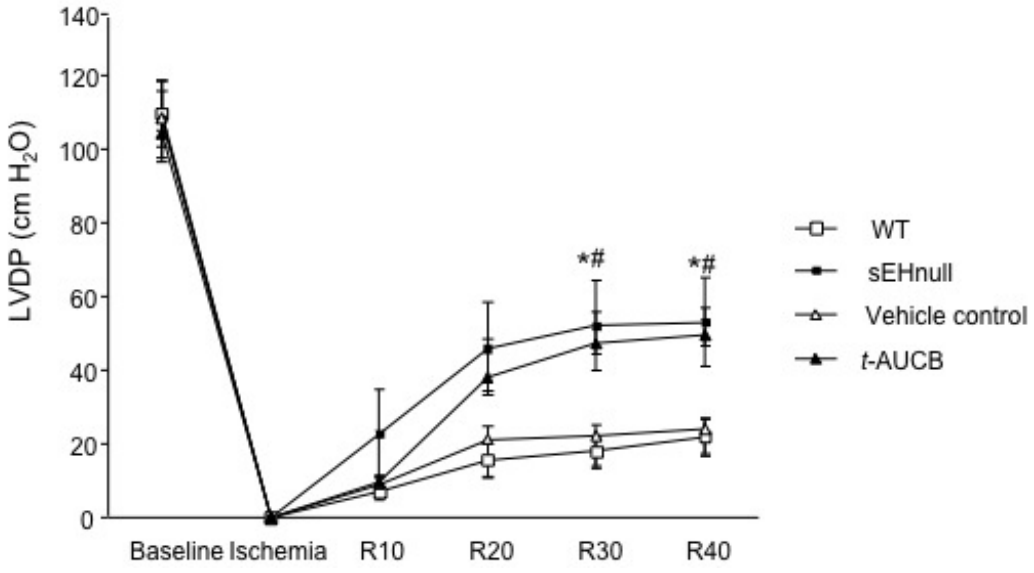

Figure 2.4 Effect of genetic or pharmacological inhibition of sEH on postischemic contractile function. LVDP in perfused hearts from WT, sEH null, vehicle control and WT treated with *t*-AUCB (0.1μM). Values represent mean ± SEM; n=5-11 per group; *, $p<0.05$ vs. WT; $^{\#}$, $p<0.05$ vs. vehicle control.

Table 2.2 Cardiac parameters for pharmacological and genetic inhibition of sEH

	WT (*n*=5)	sEH null (*n*=9)	Vehicle control (n=11)	WT + *t*-AUCB (0.1μM) (n=5)
Isolated Perfused Heart - Preischemic				
LVDP (cmH_2O) (Baseline)	114.1±9.0	112.5±10.8	112.7±7.9	108.4±3.6
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (Baseline)	3384±332	3416±507	3249±294	3361±198
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (Baseline)	-2920±257	-2809±448	-2683±188	-2786±148
HR, perfused (beats/min) (Baseline)	325±28	297±29	291±19	368±17#
Isolated Perfused Heart - Postischemic				
LVDP (cmH_2O) (R40)	22.8±4.7	55.2±12.4*	25.1±2.9	51.5±7.8#
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (R40)	587±142	1377±162*	807±92	1549±306#
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (R40)	-578±119	-1291±181*	-688±82	-1387±194#
HR, perfused (beats/min) (R40)	244±42	251±23	323±16	333±18

Hemodynamic parameters were measured in isolated-perfused hearts. Values represent mean±SEM,

* $p<0.05$ vs WT; # $p<0.05$ vs. Vehicle control. LVDP, left ventricular pressure, HR, heart rate.

2.3.3 EET mediated effects of *t*-AUCB

As previously reported, targeted deletion or pharmacological inhibition of sEH leads to increase in intracellular EET levels which can further produce cardioprotective effects following ischemia reperfusion injury.[1] To confirm the EET mediated effects of *t*-AUCB, we perfused the WT hearts with 0.1 μM *t*-AUCB in presence or absence of putative pan-EET receptor antagonist 14, 15-EEZE (10 μM). At this concentration, 14,15-EEZE has been demonstrated to inhibit EET-induced protective effects.[9] 14,15-EEZE did not affect the baseline function of the WT mice hearts. However, postischemic functional recovery of *t*-AUCB treated hearts (LVDP= 49.9 ± 7.5%) was completely abolished in the hearts co-perfused with 14,15-EEZE (LVDP= 13.3±2.5%) (Fig 2.5). To assess the effects of exogenous EET cardioprotective effects of *t*-AUCB, we perfused the WT hearts with *t*-AUCB in presence or absence of 11,12-EET (1 μM) or 14,15-EET (1 μM). Interestingly, no additive effects of exogenous EETs was observed on *t*-AUCB treated hearts (Fig 2.6). These data suggests that maximum recovery was achieved by treating the hearts with 0.1 μM *t*-AUCB and therefore addition of exogenous EETs does not show any additional effect on functional recovery.

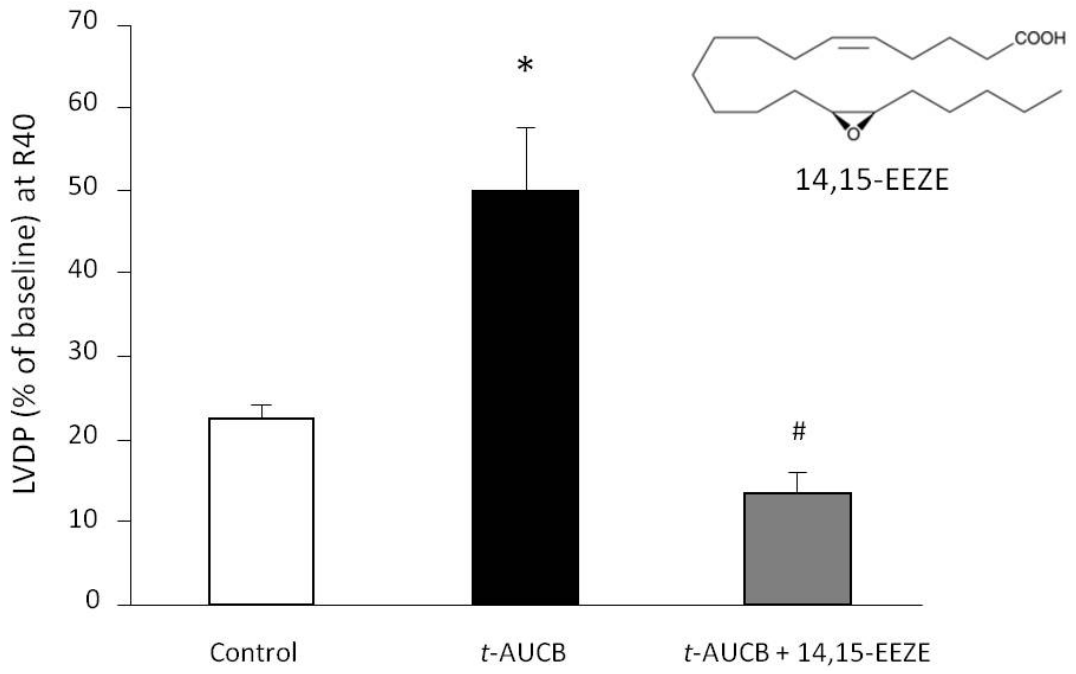

Figure 2.5 EET mediated cardioprotection in *t*-AUCB treated hearts. LVDP recovery at 40min of reperfusion in WT hearts treated with *t*-AUCB (0.1μM) in presence or absence of putative EET-receptor antagonist, 14,15-EEZE (10 μM). Values represent mean ± SEM; n=5 per group; *, $p<0.05$ vs. Vehicle control; #, $p<0.05$ vs. *t*-AUCB treated hearts.

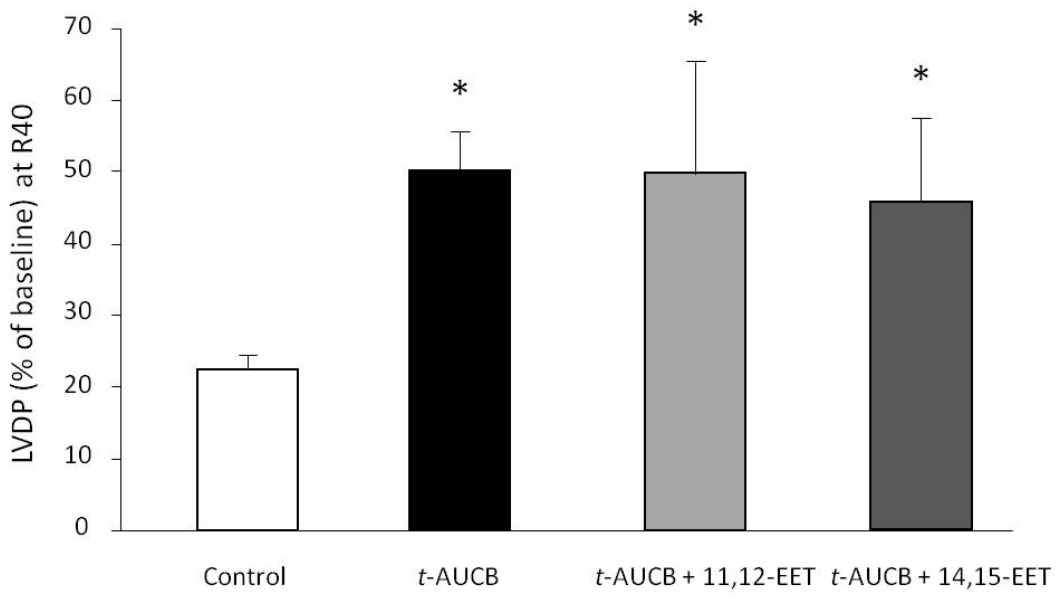

Figure 2.6 EET mediated cardioprotection in *t*-AUCB treated hearts. LVDP recovery at 40min of reperfusion as percentage of baseline. Hearts were perfused with *t*-AUCB (0.1μM) in presence or absence of exogenous 11,12-EET (1μM) or 14,15-EET (1μM). Values represent mean ± SEM; n=3-5 per group; *, $p<0.05$ vs. vehicle control.

2.3.4 PI3K mediated effects of *t*-AUCB

To determine the role of the PI3K signaling cascade in *t*-AUCB mediated cardioprotective response, we performed isolated perfused heart experiments in the presence or absence of the PI3K inhibitors wortmannin (200nM) or LY294002 (5μM). At given concentrations, wortmannin or LY294002 has been reported to inhibit EET-induced induced cardioprotection.[1] Neither inhibitor had a significant effect on baseline LVDP. Perfusion with either wortmannin or LY294002 for 5 min prior to ischemia had no effect on postischemic LVDP recovery in vehicle controls; however, both inhibitors significantly reduced the improved postischemic functional recovery in *t*-AUCB treated hearts (Fig 2.7). Thus, percent LVDP recovery at 40 min reperfusion was comparable in both the groups after treatment with either wortmannin or LY294002 (Fig 2.7). These data suggest the involvement of the PI3K cascade in the cardioprotective effects of pharmacological inhibition of sEH.

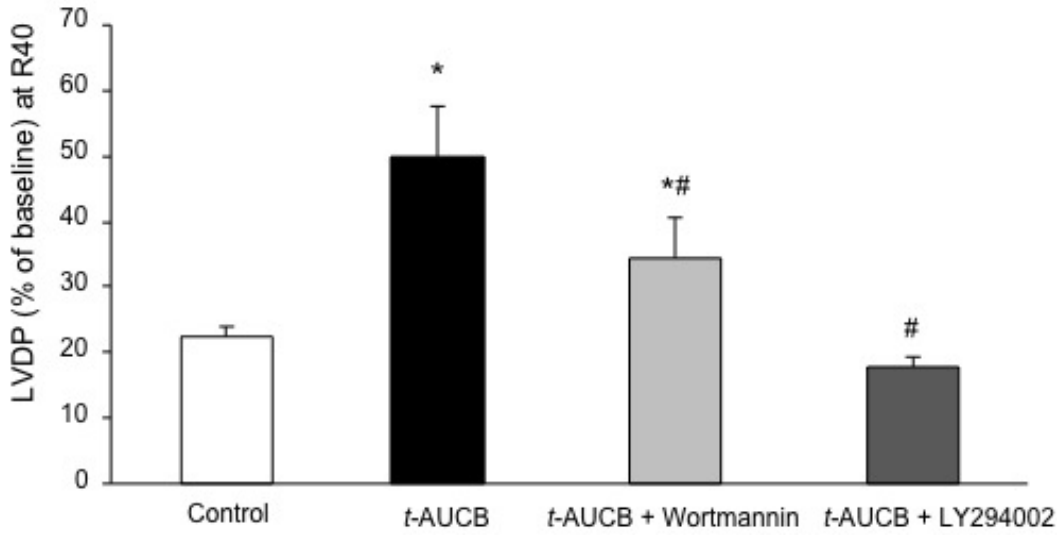

Figure 2.7 Role of PI3K in *t*-AUCB mediated cardioprotection. LVDP recovery at 40min of reperfusion as percentage of baseline. Hearts were perfused with *t*-AUCB (0.1μM) in presence or absence of PI3K inhibitor wortmannin (200nM) or LY294002 (5μM). Values represent mean ± SEM; n=5 per group; *, $p<0.05$ vs. vehicle control; $^{\#}$, $p<0.05$ vs. *t*-AUCB treated hearts.

2.4 Discussion

In this study, we report improved postischemic contractile function and reduced infarct size in isolated mice hearts perfused with a novel water-soluble and potent pharmacological sEHi, *t*-AUCB. The effects of *t*-AUCB were attenuated by the putative EET-receptor antagonist 14,15-EEZE and inhibition of PI3K. Taken together, our data suggest that pharmacological inhibition of sEH by *t*-AUCB can be protective against ischemia reperfusion injury and the effects are mediated through EETs and PI3K pathway.

sEH is a bifunctional enzyme with C-terminus hydrolase activity and N-terminus phosphatase activity.[5, 16] While the functional effects of phosphatase are not well known, much evidence implicates the importance of its hydrolase activity in cardiovascular diseases.[1, 5, 11, 15, 17-18] Two different approaches have been used to study the effects of sEH on cardiovascular system: genetic modification and pharmacological inhibition.[1, 11, 15] Genetic modulation by targeted deletion of Ephx2 gene results in lack of sEH expression and reported to produce anti-arrhythmic, antihypertensive and cardioprotective effects.[1, 19] On the other hand, sEHi causes inhibition of hydrolase activity exerting similar protective effects in the cardiovascular system.[11, 15, 20-21] Consistent with the previous observations, the data in this study indicate that both genetic and pharmacological knockout of sEH lead to a dramatic improvement on the cardiac parameters measured. However, neither

approach resulted in full return to normal function indicating that additional approaches could be helpful in restoring cardiac function. The fact that both the pharmacological and genetic knockout experiments resulted in similar improvements in function indicates that a near maximum effect for an sEHi was reached with *t*-AUCB in this system.

Small molecule inhibitors are valuable tools to study the role of sEH in the pathophysiology of cardiovascular diseases. The first generation of sEHi were epoxide-containing compounds that turned out to be substrates of EH metabolism.[16] These compounds tended to only have a transient inhibitory effect *in vitro* and were not effective *in vivo*.[16, 22] EH inhibitory properties were shown with urea, amide and carbamate functionalities as the central pharmacophore. The ureas were in general the most active with similar R groups, but amide and carbamate derivatives with optimized substituents can be as active as ureas.[16] The next generation of sEHi demonstrated that 1,3-disubstituted urea derivatives were stable and more potent sEHi than previous compounds.[16, 23] *t*-AUCB has a lipophilic adamantane on the N of the urea and a bicyclic system with a polar ether and acid on the N' position which dramatically increase water solubility.[24-26] Formation of hydrogen bonds and salt bridges between urea functionality and active cites of sEH results in inhibition of hydrolase activity.[16] sEH has a high V_{max} and low K_M for endogenous epoxides of arachidonic and linoleic acid which results in diminished biological activity. Emerging evidence demonstrates that sEHi are potential therapeutic

compounds for the treatment of several diseases. *cis*-isomer of *t*-AUCB (*cic*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid; *c*-AUCB) has recently been demonstrated to attenuate monocrotaline induced pulmonary hypertension in rats.[27] Xu et al., has reported importance of sEH inhibition prevented and reversed cardiac hypertrophy using 1-adamantan-3-(5-(2-(2-ethylethoxy)ethoxy)pentyl)urea, AEPU.[28] Similarly, 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS) can block angiotensin-II induced cardiac hypertrophy.[20] 1,3-disubstituted urea sEHi such as AUDA and AUDA-BE have demonstrated protective effects against ischemia reperfusion injury in the micromolar range.[11, 15] In the present study, *t*-AUCB exerted cardioprotective effects by inhibiting sEH activity at nanomolar concentrations. Apart from urea based sEHi, non-urea derivatives of potent sEHi have also been reported; however, no study has demonstrated *in vivo* effectiveness of these compounds.[29-30]

Cardioprotective effects towards ischemia reperfusion injury by sEHi compounds have only been demonstrated with AUDA and AUDA-BE.[11, 15] These sEHi compounds have several drawbacks which include limited oral bioavailability, instability due to rapid metabolism and poor physical properties such as low water solubility.[18] With very careful formulation AUDA and its salts and esters can be orally administered for in vivo use, but the new generation of sEHi such as *t*-AUCB are dramatically easier to administer and have longer half lives.[5, 11, 15, 18, 31] *t*-AUCB

(IC_{50} = 1.3 nM) is a more potent inhibitor of sEH than AUDA (IC_{50} = 3 nM) and AUDA-nBE (IC_{50} = 7 nM), while it is equipotent to TPAU (IC_{50} = 1.1 nM) and *c*-AUCB (IC_{50} = 0.89 nM).[26] Liu et al. compared AUDA-BE to *t*-AUCB for total epoxide and diols in plasma of LPS treated mice. Both, AUDA-BE and *t*-AUCB, significantly reduced DHET formation increasing the epoxide to diol ratio. However, *t*-AUCB was able to increase the EET/DHET ratio to similar levels of AUDA-BE at a 100 times lower dose.[24] *t*-AUCB has better oral bioavailability and metabolic stability as higher area under concentration time curve (AUC_t) and longer elimination half life ($t_{1/2}$) (AUC_t = 155 μM×min; $t_{1/2}$ > 1400 min) compared to AUDA (AUC_t = 24 μM×min; $t_{1/2}$ = 575 min) and AUDA-BE (AUC_t = 16 μM×min; $t_{1/2}$ = 260 min).[18, 24, 26, 31] Similarly, pharmacokinetic studies done in canine model revealed that *t*-AUCB was found in the circulation for 1 day following oral administration.[18] The improved physical and pharmacokinetic properties of *t*-AUCB make it an attractive therapeutic agent and experimental probe for increasing beneficial EETs.

Inhibition of sEH decreases metabolism of EET and thereby exerts protective effects against injury incurred following myocardial ischemia reperfusion and ischemic stroke.[1, 11, 15, 17] Increased EETs in the heart activate various cardioprotective pathways leading to improved contractile function and reduced damage to the heart.[1, 11, 13] The cardioprotective effects of EETs and sEHi can be blocked by simultaneous treatment with putative EET receptor antagonist, 14,15-EEZE.[1, 9-11]

Consistent with these observations, our data suggest EETs mediate the protective effects of *t*-AUCB, as the improved post ischemic functional recovery was abolished by co-perfusion with 14,15-EEZE. It was surprising that addition of 11,12-EET to this system did not further increase cardiac function. This may be the result of sufficient amounts of EET, other omega-6 or 3 epoxides free or esterified in cardiac tissue, or the synthesis rate of epoxides is high enough that supplementation is not needed. Recent evidence suggests that EETs can activate PI3K/Akt signaling pathway which leads to inhibition of GSK-3β and reduces the damage to mitochondria.[1, 10] Dhanasekaran et al. reported EET-mediated activation of multiple antiapoptotic targets through PI3K/Akt survival signaling.[31] Moreover, inhibition of PI3K pathway by wortmannin or LY294002, abolished the improved postischemic functional recovery of sEH null mice.[1] Similarly, our results show significant reduction in postischemic functional recovery of hearts co-perfused with *t*-AUCB and PI3K inhibitor wortmannin or LY294002, which confirms the involvement of PI3K survival signaling.

2.5 Conclusion

In conclusion, we report improved postischemic contractile function and reduced infarct size by treatment with a sEHi, *t*-AUCB. Moreover, the cardioprotective effect was attributed to EETs and the PI3K survival pathway. The increased potency and water solubility of *t*-AUCB over other sEHi suggest this compound may serve as a potential therapeutic agent in myocardial ischemia reperfusion injury and potentially a valuable drug for treatment ischemic heart diseases.

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Chapter 3

Role of B-type Natriuretic Peptide in Epoxyeicosatrienoic Acid Mediated Improved Postischemic Recovery of Heart Contractile Function

[3]A version of this chapter has been published: Chaudhary KR, Batchu SN, Das D, Suresh MR, Falck JR, Graves JP, Zeldin DC, Seubert JM. Role of B-type natriuretic peptide in epoxyeicosatrienoic acid-mediated improved post-ischaemic recovery of heart contractile function. *Cardiovasc Res. 2009 Jul 15;83(2):362-70.*

3.1 Introduction

Arachidonic acid (AA), an essential polyunsaturated fatty acid found esterified to membrane phospholipids, can be metabolized by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP) epoxygenases to numerous products collectively termed eicosanoids, which have important cellular functions.[1-2] Ischemic events activate cytosolic phospholipase A_2 which will release AA from glycerophospholipids stores.[3] AA can then be metabolized by various CYPs to four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs), all of which are biologically active.[2] Modulation of EET levels can occur via reincorporation into phospholipid membranes or by β-oxidation to smaller reactive epoxides;[4-5] however, the predominant pathway occurs through metabolism to inactive vicinal diols compounds by epoxide hydrolases.[1, 6] Two major epoxide hydrolases are found in mammalian tissues, microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH or *Ephx2*).[5]

Accumulating evidence indicates that EETs have important functional roles in ischemia-reperfusion injury. They activate various stress response systems, such as the phosphoinositide 3-kinase (PI3K) pathway; enhance membrane ion channel activity, such as sarcolemmal ($sarcK_{ATP}$) and mitochondrial ($mitoK_{ATP}$) ATP-sensitive K^+ channels; and improve postischemic recovery of left ventricular function.[7-12] Evidence indicates that EET-mediated cardioprotective signals target glycogen synthase

kinase-3β (GSK3β), a key protein involved in cellular function and signaling essential to cardioprotection.[13-14] Regulated via phosphorylation, GSK3β acts as a central convergence point targeting upstream signaling to mitochondria.[13, 15] Published reports indicate that multiple protective pathways converge upon GSK3β prior to effecting mitochondrial function.[7, 13, 16] Alteration in the production and/or elimination of EETs may affect steady-state cellular levels of these bioactive eicosanoids *in vivo* and could potentially influence cardiac function.

B-type or brain natriuretic peptide (BNP) is becoming a valuable biomarker for cardiovascular disease that bears diagnostic, prognostic and therapeutic importance in congestive heart failure, arrhythmias and acute myocardial infarction.[17-18] Evidence indicates that BNP can attenuate ischemic-reperfusion injury in animal models but the underlying mechanism is unknown.[19-20] Isolated perfused rat heart studies suggest that increased BNP expression at baseline and release of peptide in the coronary effluent during reperfusion are attributed to wall stretch and acute ischemic injury.[21-22] Cardioprotective effects of BNP correlate with elevated cGMP and NO levels which can be abolished by inhibiting the mitoK_{ATP} channel.[20-21] The specific involvement of BNP and mitoK_{ATP} opening in cardioprotection is largely unknown. The anti-ischemic profile of natriuretic peptides and correlation to mitoK_{ATP} suggest

that endogenous BNP may be an attractive target for cardioprotection and may warrant further investigation.

Recently, we reported that mice with the targeted disruption of the *Ephx2* gene had enhanced postischemic recovery of left ventricular function, which was mediated by activation of the PI3K pathway and K^+ channels.[10] In the present study, we demonstrate that EET-mediated cardioprotection involves increased expression of BNP. Moreover, our data suggest a role for PKCε and GSK3β in integrating EET-mediated effects to the mitochondria. Taken together, these data merge two endogenous cardioprotective mediators, EETs and BNP, provide a novel mechanism for cardioprotection and suggest a potential target for therapeutic intervention.

3.2 Material and Methods

3.2.1 Chemicals

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Mississauga, Canada). The anti-His_6 MAb was purchased from Novagen Inc. (Madison, USA). Glutathione (reduced and oxidized), sodium deoxycholate, l-arginine, HRPO conjugated goat anti-mouse, urea and other general molecular biology grade reagents were purchased from Sigma (Oakville, Canada). Ni–NTA agarose, plasmid DNA isolation and gel extraction kits were obtained from Qiagen (Mississauga, Canada).

3.2.2 Animals

A colony of mice with targeted disruption of the *Ephx2* gene (sEH null), originating from Darryl Zeldin (NIH/NIEHS) and backcrossed onto a C57BL6 genetic background for more than 7 generations, is maintained at the University of Alberta. C57BL6 mice and New Zealand White rabbit were purchased from Charles River Laboratories (Pointe Claire, PQ). All experiments used male and female mice aged 3-5 months, weighing 20-34 g and were treated in accordance with the guidelines of Health Science Laboratory Animal Services (HSLAS), University of Alberta. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2.3 Isolated Heart Perfusions

Mouse hearts were perfused in the Langendorff mode as previously published.[14, 23] Hearts were perfused in the Langendorff mode in retrograde fashion at constant pressure (100cmH_2O). Continuously aerated (95%O_2/5%CO_2) modified Krebs-Henseleit buffer (NaCl 120mM; KCl 4.7mM; $CaCl_2$ 1.75mM; $MgSO_4$ 1.2mM; KH_2PO_4 1.2mM; $NaHCO_3$ 25mM; Na-pyruvate 2mM; glucose 10mM) was used to perfuse the isolated hearts. A balloon-tipped catheter was inserted into the left ventricle through the left atrium. Pressure was recorded using pressure transducer connected to the catheter. A PowerLab system (AD Instruments) was used to process data. Hearts were perfused with buffer for 40min of stabilization period and then subjected to 20 or 30min global no-flow ischemia, followed by 40min reperfusion. Some isolated sEH null and WT hearts were treated with the PI3K inhibitor, LY294002 (5 μM) or the putative EET receptor antagonist 14,15-EEZE (100nM) prior to ischemia. Hearts were perfused with rBNP (10 nM) in presence or absence of A71915 (NPR-A antagonist, 50-100 nM) throughout the reperfusion period. Percent of left ventricular developed pressure at 40min of reperfusion, as compared to base line, was taken as a marker for functional recovery. Stock solution for BNP, A71915, 14,15-EEZE, LY294002 and wortmannin were prepared in PBS, ddH2O, 100% ethanol, DMSO and DMSO, respectively. Appropriate vehicle controls were performed for each group.

3.2.4 Infarct size analysis

To determine the amount of infarction, following 40 min of stabilization period and 30 min global no-flow ischemia, hearts were reperfused for 2 h. After 2 h reperfusion, hearts were perfused with 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in Krebs-Henseleit buffer at 37 °C for 10 min, then fixed in formalin and cut into thin cross-sectional slices. The area of infarction was quantified by measuring stained (red, live tissue) and unstained (white, necrotic) regions using Image J (NIH, USA).

3.2.5 Gene Expression

Quantitative real-time PCR (qPCR) analysis of natriuretic peptide precursor type B (NM_008726) expression was performed in hearts from naïve mice and isolated perfused experiments. Total RNA was isolated using an RNeasy Midi kit (Qiagen, Valencia, CA) and concentrated using a Microcon YM-30 column (Millipore, Billerica, MA). A formaldehyde agarose gel containing ethidium bromide was used to assess the quality of the RNA. mRNA from individual hearts were treated with DNase I and then 1 μg was used to prepare cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). cDNA levels were detected using qPCR with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each individual sample was normalized to the RNA expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within the same heart. Fold

expression change was determined by quantitation of cDNA from target (perfused) samples relative to a calibrator sample (naïve) of the same genotype. To determine this normalized value, $2^{-\Delta\Delta CT}$ values were compared between target and calibrator samples, where ΔCt = target gene (crossing threshold) Ct - GAPDH Ct, and $\Delta\Delta$Ct = $\Delta Ct_{sEHnull}$ - ΔCt_{WT}.[24] All the RNAs from WT and sEH null hearts were made on the same day and also analyzed together on the same PCR plate.

3.2.6 Construction of plasmid (pDS18BNP)

BNP nucleotide sequence (BNP, NM_008726) was codon optimized for E. coli expression and chemically synthesized from GENEART, Germany. The codon optimized BNP gene containing plasmid obtained from GENEART Inc. and the expression vector pBM802 were digested with NdeI and EcoRI, gel purified and ligated. The ligation mixtures were transformed in E. coli top 10 cells and bacterial colonies were analyzed by plasmid DNA isolation and restriction digestion fragment mapping.

3.2.7 Recombinant clones analysis

Single bacterial colonies (six different clones) were cultured in 2 mL TB medium containing 5 µg/ml of tetracycline (TB/Tet^5) and were incubated overnight at 37 °C with shaking (250 rpm). The overnight culture was diluted to 1:100 in 10 mL fresh TB/Tet^5 medium and grown at 37 °C. The bacterial culture was induced when the optical density (OD_{600nm}) reached approximately 0.5–0.6 with arabinose [0.2% (w/v)], whereas in

control sample arabinose was not added. The bacterial culture was allowed to grow overnight (~16 h) at 37 °C. The bacterial culture of test and control samples were harvested by centrifugation at 5000g for 10 min at 4 °C and the total cell lysate was prepared using lysis buffer. Total cell protein was analyzed by SDS–PAGE using 10% polyacrylamide gel. The protein gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 30% methanol. Western blotting was performed using anti-His_6 antibody to confirm expression of His_6 tagged recombinant BNP (rBNP).

3.2.8 Optimization of expression condition

The expression of the BNP protein was optimized for time of incubation and inducer (arabinose) concentrations. Bacterial growth conditions were similar as described above. For arabinose dose optimization, the bacterial culture was induced with different concentrations of arabinose [0.2%, 0.02%, 0.002%, 0.0002% and 0.00002 (w/v)] and allowed to grow overnight (~16 h) at 37 °C. For time optimization, the bacterial culture was induced with arabinose [0.2% (w/v)] and allowed to grow for 0 h, 2 h, 4 h and overnight (~16 h) at 37 °C. Total cell protein from each optimization experiment were analyzed by SDS–PAGE and Western blot to select the ideal condition for optimum protein expression.

3.2.9 Medium scale expression and purification of BNP protein

The plasmid DNA of best producer clone was transformed in E.coli Rosetta by heat shock method and plated on TB medium containing 5 µg/ml tetracycline and 34 µg/ml chloramphenicol (TB/Tet^{5}/$Chlor^{34}$) as E.coli Top10 was not compatible with medium scale production. A single bacterial colony was inoculated in 10 ml TB/Tet^{5}/$Chlor^{34}$ medium and allowed to grow overnight at 37 °C shaker. The overnight culture was diluted (1:100) in fresh 4 × 1 L TB/Tet^{5}/$Chlor^{34}$ medium and grown at 37 °C until an OD_{600nm} of 0.5–0.6 was reached. Expression was done by optimized conditions as described in the previous section. Induction was initiated by adding 0.2% (w/v) arabinose and bacterial culture was incubated for 16 h with vigorous shaking at 37 °C. Bacterial culture was harvested by centrifugation at 5000g for 20 min at 4 °C and total cell protein from induced and uninduced culture was analyzed by SDS–PAGE and Western blot probed with anti-His6 MAb.

3.2.10 Purification of inclusion bodies (IB)

The purification of inclusion bodies was done according to previously published method.[25] Briefly, 4 g of bacterial wet pellet from 4 L bacterial culture was suspended in 40 mL PBS (10 mL PBS per g of pellet) and completely lysed by passing through a French Press (20,000 psi). The total cell lysate was clarified by centrifugation at 27,000g for 30 min at 4 °C and supernatant was collected as total soluble protein. The

pellet was resuspended in lysis buffer (Lysis buffer 50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA) and then 2% sodium deoxycholate was added. The mixture was incubated at room temperature for 30 min with gentle shaking and centrifuged at 27,000g for 30 min at 4 °C. The pellet was resuspended in lysis buffer and washed thrice at 27,000g for 20 min at 4 °C to completely remove sodium deoxycholate. Inclusion bodies were solubilized in denaturing buffer B (Buffer B: 8 M urea, 100 mM NaH2PO4, 10 mM Tris–Cl, pH 8.0) for 1 h at room temperature with gentle shaking. Solubilized denatured BNP proteins from insoluble materials were separated by centrifugation at 27,000g for 30 min at 4 °C. Final yield of solubilized denatured protein was determined by protein assay using BSA as the standard protein. A Ni–NTA column was prepared by loading the Ni–NTA agarose on a plastic column (Bio-Rad) and equilibrated with 10 bed volumes of buffer B. Twenty milligrams of solubilized denatured BNP protein was loaded on the column and the column was washed with 5–10 bed volumes with buffer C (Buffer C: 8 M urea, 100 mM NaH2PO4, 10 mM Tris–Cl, pH 6.3). After complete wash, bound protein was eluted with buffer D (Buffer D: 8 M urea, 100 mM NaH2PO4, 10 mM Tris–Cl, pH 5.9) and buffer E (Buffer E: 8 M urea, 100 mM NaH2PO4, 10 mM Tris–Cl, pH 4.5). All the eluted fractions were analyzed by SDS–PAGE prior to refolding.

3.2.11 Refolding

Protein assay was done to measure the amount of protein eluted from the Ni–NTA column and it was estimated ~0.1- 0.3 mg/mL with a total amount of ~4 mg. Refolding was done in three different concentrations to evaluate the best refolding condition. The eluted protein was adjusted to 100, 75 and 50 µg/ml with refolding Tris-arginine buffer and refolding was done by dialysis in TA buffer (50 mM Tris, pH 8.0, 0.4 M l-arginine) in the presence of 1.0 mM GSH (glutathione, reduced), 0.1 mM GSSG (glutathione, oxidized) for 3 days with two changes at 4 °C. Final dialysis was done in PBS pH 7.4 at 4 °C.

3.2.12 Development of rabbit anti-BNP polyclonal antibody

Rabbit anti-BNP polyclonal antibody was produced using a female rabbit (NZW). Rabbit (NZW) was obtained from Health Sciences Laboratory Animals Services (HSLAS) of the University of Alberta, Edmonton, Canada. Animal treatment and care were carried out according to the University of Alberta guidelines. Female Rabbit (NZW) was immunized subcutaneously on day 0 and 14 with rBNP antigen (100 µg) emulsified with complete and incomplete Freunds adjuvant (1:1) respectively. Rabbit was then further immunized with 50 µg antigen emulsified with incomplete Freunds adjuvant (1:1) on day 28 and test bleeding was done on day 42. Indirect ELISA was performed to determine the immune response of the polyclonal serum against rBNP. Last booster injection was given on day 56 with rBNP (50 µg) emulsified with

incomplete Freunds adjuvant (1:1) and terminal bleeding was performed on day 62.

3.2.13 Purification of anti-BNP polyclonal antibody

Anti-BNP polyclonal antibody was purified using protein-G affinity chromatography. Serum (30 mL) was used for purification. Each 10 mL of serum was diluted up to 100 mL with PBS (pH 7.3). Diluted serum was then purified using protein-G affinity chromatography column (Sigma, USA). Column was first washed with autoclaved water followed by equilibrium with PBS. Subsequently, sample was run through the column and wash was performed using PBS to remove access of serum proteins. Elution was done with 0.1 M glycine (pH 2.8). Purified antibody fractions were collected in glass tube containing 200 μL of Tris buffer (pH 9.0) to normalize the acidic pH of the eluent. The purified fractions of anti-BNP polyclonal antibody were stored at -80° C until further use. Specificity of the anti-BNP polyclonal antibody was studied by comparing the mouse protein sequences of ANP (NP_032751) and CNP (NP_035063) with BNP (NP_032752). Sequence alignment analysis performed using EMBOSS (European Bioinformatics Institute, Cambridge, UK) revealed less than 30% identity between BNP and ANP or CNP.

3.2.14 Antibody titer

For antibody titer, NUNC Maxisorp™ microwell plate (Thermo Scientific, Rochester, NY, USA) were coated with 100 μg rBNP overnight 4°C. Purified antibody was diluted to 500, 250, 100 and 50 ng/mL in PBS.

rBNP coated plate was washed with PBST (0.1% Tween 20 in 1× PBS, pH 7.3) followed by 1 hr incubation with diluted anti-BNP at room-temperature. After washing with PBST, goat anti-rabbit MAb (1:10000, 100μL) was added to each well and allowed to incubate for 1 hr at room-temperature. Subsequently, washing was performed with PBST and 100 μL of TMB substrate was added to each well. Following incubation of 10-30 min with shaking, plates were read at 620 nM.

3.2.15 Tissue Homogenization and Sub-cellular Fractionation

For preparation of subcellular fractions, tissues were minced and homogenized in homogenization buffer containing (sucrose 250 mM, Tris/HCL 10 mM, EDTA 1 mM, sodium orthovanadate 1 mM, sodium flouride 1 mM, aprotinin 10 µL/L, leupeptin 2 µL/L, pepstatin 100 µL/L). The homogenate was centrifuged at 700 × g for 10 min to remove the debris and the supernatant was again centrifuged to 10,000 × g for 20 min at 4 °C. The resulting supernatant (S-9) fraction and pellet were separated and the pellet was used as the mitochondrial fraction. The supernatant obtained from further centrifugation of the S-9 fraction at 100,000 × g for 1 h at 4 °C was used as the cytosolic fraction. The protein concentration of these fractions was determined colorimetrically using a Bio-Rad BCA protein assay kit using bovine serum albumin as a standard.

3.2.16 Immunoblot Analysis

SDS-polyacrylamide gel electrophoresis of cytosolic or mitochondrial proteins (100 µg and 25 µg, respectively) were performed

with 4% stacking and 12% separating gels. Following transfer of the separated proteins to nitrocellulose membranes (Boi-Rad), blots were blocked with 5% skimmed milk powder dissolved in TBS (0.1 M Tris-Cl, 0.9% NaCl, pH 7.4). After blocking, blots were washed three times for 10 min with TBS-T (TBS containing 0.1% Tween 20) and incubated with primary antibodies to PKCε, phospho-PKCε (p^{Ser729}PKCε), GSK3β, phospho-GSK3β (p^{Ser9}GSK3β), Akt, phospho-Akt (p^{Ser473}Akt), GAPDH (Cell Signaling Technology, Inc., Danvers, MA) or prohibitin (Fitzgerald, Concord, MA). GAPDH and prohibitin were used as loading controls for cytosolic and mitochondrial fraction, respectively. All primary antibodies were prepared in TBS containing 1% BSA to reduce non-specific binding. The blots were washed for three times for 15 min with TBS-T. Following this, appropriate horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibody in 2% skimmed milk/TBS-T was incubated with the blot. Washing was performed as for the primary antibody. Chemiluminescent detection was carried out using ECL reagent (GE health care, UK) and exposure to x-ray film. The exact same blot was used to assess both total and phosphorylated protein, for respective proteins of interest. Two separate blots were utilized to validate results. The first blot is probed with phospho-protein, stripped and then re-probed with the total protein. On our validation step, the second blot is probed in the reverse procedure. Stripping was performed using Stripping buffer (Thermo Scientifics, IL) as per manufactures protocol. The band images

were acquired using the HP scanjet 7400c and relative band intensities were assessed by densitometry using Image J (NIH, USA). All blots analyzing protein expression and loading control were scanned at the exact same time. Protein expression in vehicle treated controls were taken as 100% and compared with treated group.

3.2.17 Statistical Analysis

Values expressed as mean ± standard error of mean (SEM). Statistical significance was determined by the unpaired Student's t-test and one-way ANOVA. To determine whether significant difference exists between the groups, both Duncan's and Newman-Keuls post-hoc tests were performed. Values were considered significant if $p < 0.05$.

3.3 Results

3.3.1 Increased Nppb mRNA expression in sEH null mouse hearts

qPCR analysis of preproBNP (Nppb) mRNA revealed no significant differences in cardiac expression between naïve sEH null mice and wild-type (WT) littermates (Fig 3.1). Similarly, aerobically perfused WT and sEH null hearts had similar levels of Nppb mRNA (Fig 3.1). However, significant differences between WT and sEH null hearts, Nppb mRNA levels were observed following global ischemia and reperfusion (Fig 3.1). To determine if the increased Nppb mRNA levels in sEH null hearts was mediated by EETs, we conducted experiments in the presence of 14,15-EEZE. This putative pan-EET receptor antagonist caused a significant reduction in postischemic Nppb mRNA levels in sEH null hearts but had no effect on WT hearts (Fig 3.1). Perfusion of hearts with LY294002, a PI3K inhibitor, did not affect the increased postischemic expression of Nppb mRNA in sEH null or in WT hearts (Fig 3.1).

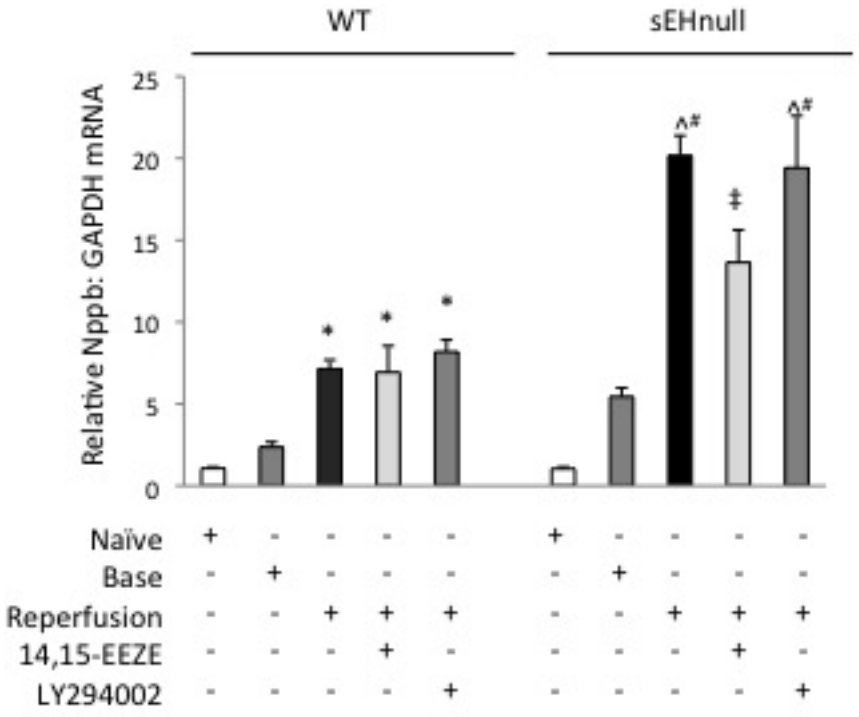

Figure 3.1 Nppb mRNA expression following I/R. qPCR data showing relative expression of Nppb to GAPDH mRNA in non-perfused (naïve) WT and sEH null mouse hearts, during 20min aerobic baseline perfusion (base) and at 40min postischemic reperfusion (reperfusion). Hearts were administered vehicle, 14,15-EEZE (1μM) or LY294002 (5μM). Values represent mean ± SEM; n=3; *, $p<0.05$ vs. naïve WT; ^, $p<0.05$ vs. vehicle treated WT; #, $p<0.05$ vs. naïve sEH null; ‡, $p<0.05$ vs. vehicle treated controls of the same genotype.

3.3.2 BNP gene cloning and small scale expression

To investigate whether increased Nppb mRNA expression in sEH null mice results in increased BNP protein expression and whether BNP plays a role in EET-induced cardioprotection, antibody against mouse BNP was required. Therefore, using E.Coli expression system, recombinant mouse BNP (rBNP) was produced and that was used to develop polyclonal antibody against mouse BNP.

The E. coli codon optimized BNP cDNA was cloned into plasmid pBM802 in the reading frame with His6 tag at the C-terminal and controlled by the arabinose pBAD promoter to increase protein expression levels in inclusion bodies in E. coli. This new construct was termed as pDS18BNP (Fig 3.2a). The correct size recombinant clones were selected for protein expression. The plasmid containing full-length BNP gene was isolated for expression. Expression results showed that all the BNP clones selected were expressing the target protein of approximately 16 kDa at different levels when analyzed by SDS–PAGE where as in control sample (without arabinose) there was no expression of the target protein (Fig. 3.2b). The expression of BNP protein was confirmed by Western blot probed with anti-His6 MAb (Fig. 3.2b). The best BNP clone was chosen for the expression optimization and further studies.

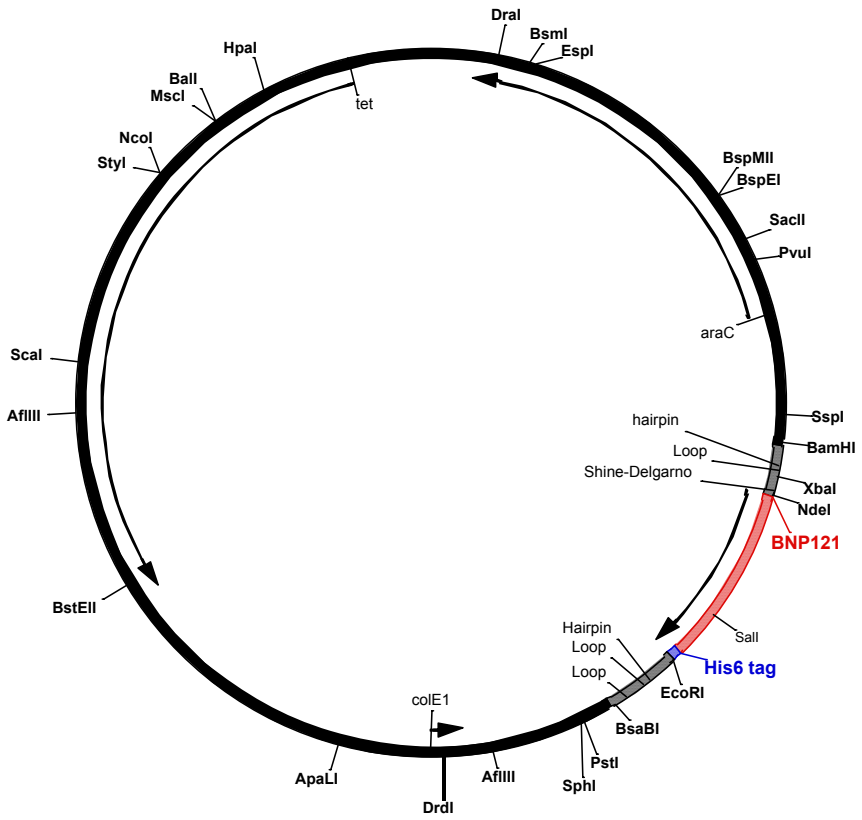

Figure 3.2a BNP plasmid construction (pDS18BNP). Full length BNP gene with *His*$_6$-tag in *E. coli* expression vector.

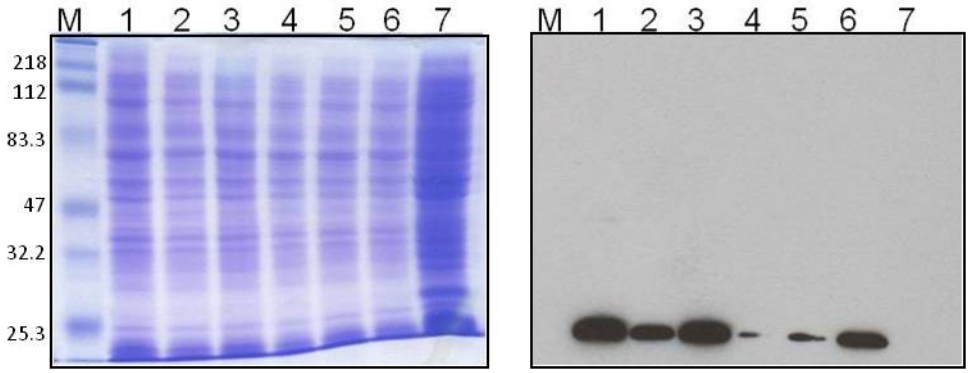

Figure 3.2b Selection of clone. SDS–PAGE analysis of different BNP clones expression in E. coli. Lane M: standard protein molecular weight markers, lanes 1–6: clone# 1, 2, 3, 4, 5 and 6, respectively, lane 7: control.

3.3.3 Optimization of expression condition and medium scale expression

The BNP protein was successfully expressed as inclusion bodies in E. coli. The optimal conditions for BNP protein expression at 37°C were 0.2% (w/v) arabinose concentration (Fig. 3.3a) and 4 h induction time (Fig. 3.3b). However, there was no difference in protein expression between 4 h induction and overnight (~16 h) induction therefore either 4 h or overnight incubation can be performed for medium scale expression.

The bacterial expression vector pBM802 was designed for high-level expression of recombinant protein in E. coli as inclusion bodies. The medium scale expression of the rBNP was performed and there was high-level expression in E. coli when analyzed by SDS–PAGE (Fig. 3.3 and Fig 3.4). Inclusion bodies were prepared from the bacterial pellet by a French Press. Following complete bacterial cell lysis, the insoluble inclusion bodies were separated from total soluble protein by centrifugation. The pellet was washed with sodium deoxycholate and subsequently washed with lysis buffer to remove any sodium deoxycholate. The final yield of denatured soluble inclusion bodies was estimated to be approximately 1mg/L of initial bacterial culture. The purity of the inclusion bodies along with different washes was analyzed by SDS–PAGE. (Fig 3.4 and Fig 3.5a). Inclusion bodies demonstrated single band at the expected molecular weight (16 kD), while the washes and control samples did not demonstrate any band (Fig 3.5a).

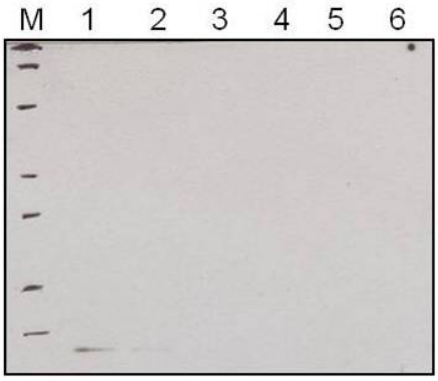

Figure 3.3a Optimization of inducer concentration. SDS–PAGE analysis of arabinose concentration dependent rBNP protein expression. Lane M: standard protein molecular weight markers, lanes 1–5: bacterial cultures were induced with 0.2%, 0.02%, 0.002%, 0.0002%, 0.00002% arabinose (w/v), respectively, lane 6: control.

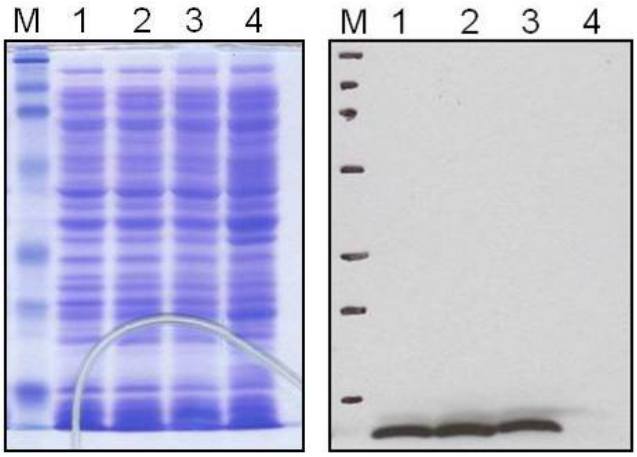

Figure 3.3b Optimization of time of induction. SDS–PAGE analysis of time dependent rBNP protein expression. Lane M: standard protein molecular weight markers, lane 1: 16 h, lane 2: 4 h, lane 3: 2 h, lane 4: 0 h.

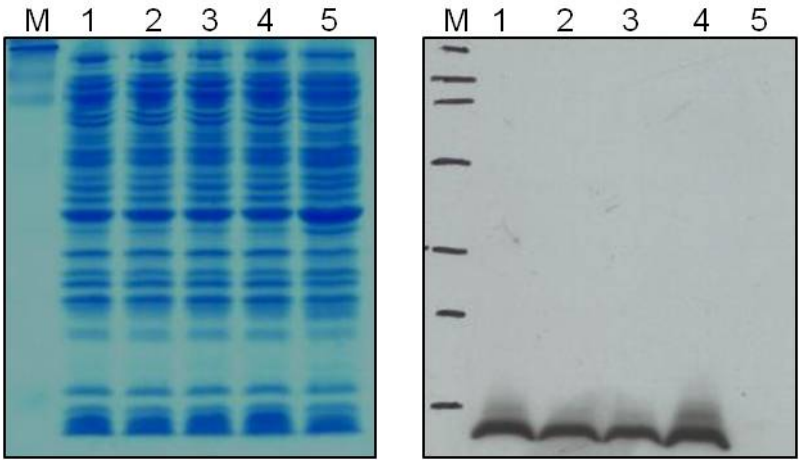

Figure 3.4 Medium scale production of rBNP. SDS–PAGE analysis of medium scale production of BNP protein. Lane M: standard protein molecular weight markers, lanes 1–4: bacterial culture flask 1–4 (f1–f4), lane 5: control.

3.3.4 Purification of rBNP

The rBNP protein was predominantly expressed in E. coli as inclusion bodies (Fig 3.4 and Fig 3.5a). The inclusion bodies were isolated from bacterial shake flask culture and purified by IMAC chromatography under denaturing conditions. The purity of the eluted rBNP protein was analyzed by SDS–PAGE prior to refolding (Fig. 3.5A). Renaturing conditions, protein concentrations in the refolding buffer as well as suitable buffer compositions are important to simulate correct folding, formation of the proper disulfide bond and proper association of different domains. The refolding step was done for 3 days by dialysis and any aggregate formed during refolding was removed by centrifugation. The supernatant was collected as refolded rBNP for further use. The purified and refolded rBNP was analyzed by SDS-PAGE and western blotting and purity of the refolded protein was evident from the single band in the gel as well s in the blot (Fig 3.5a). Thus the in vitro refolding proved to be successful in recovering soluble protein expressed in E. coli as inclusion bodies. The purity of the refolded BNP protein was evident from the SDS–PAGE and Western blot (Fig. 3.5a) with a single band of approximate molecular weight of 16 kDa. Followed by purification and refolding, stability of rBNP at 4°C was studied for 7-days by western blotting and protein assay. rBNP was stable for 4 days, while on the day-5 protein aggregation was observed. Significant decrease in the amount of protein was observed on day-7 (Fig 3.5b). Moreover, freeze-thawing had negative impact on the

stability of rBNP and that resulted in aggregation of the recombinant protein. Based on these observations, all the experiments using rBNP were performed within first two days after refolding and freeze-thawing was avoided.

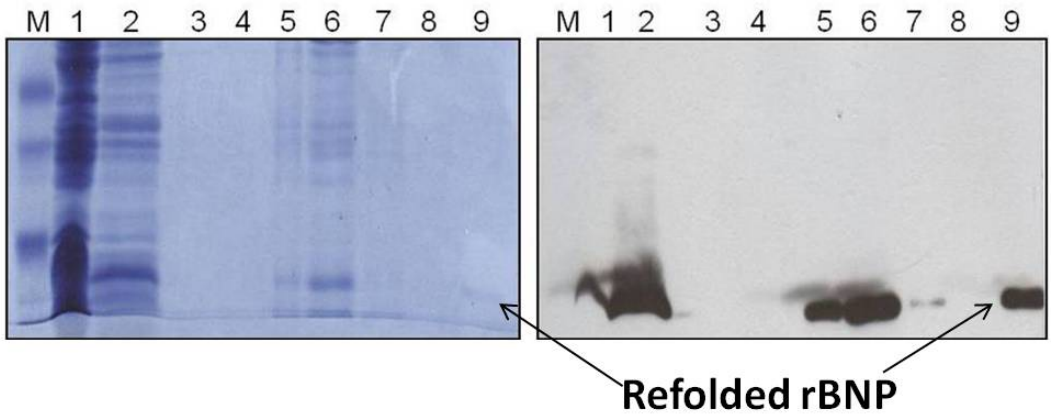

Figure 3.5a IMAC purification and refolding. SDS–PAGE analysis of IMAC purified BNP protein. Lane M: standard protein molecular weight markers, lane 1: cell lysate, lane 2: unbound protein, lanes 3 and 4: washes, lanes 5, 6 and 7: elutions, lane 8 and 9: refolded BNP.

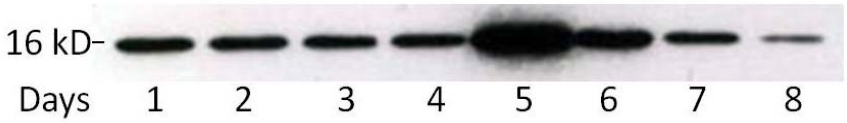

Figure 3.5b Stability of rBNP. rBNP was stable for 4-days in PBS at 4°C. At day 5 protein aggregation was observed.

3.3.5 Development of anti-BNP and characterization

Anti-BNP polyclonal antibody was developed against mouse BNP, purified and characterized for western blotting (Fig 3.6, Fig 3.7a and Fig 3.7b). Test bleed was performed on day 42 of immunization and indirect ELISA was performed to determine the immune response of the polyclonal serum against rBNP. Following the booster dose terminal bleed was performed and polyclonal serum was purified using protein-G affinity chromatography. Antibody titer was performed to determine the immune response of the polyclonal serum and purified polyclonal anti-BNP antibody (Fig 3.6 and Fig 3.7a). Polyclonal serum was able to detect the rBNP at the dilution as low as 1:10,000 (Fig 3.6) and the response was higher than that of the test bleed sample (data not shown). On the other side, the lowest concentration of purified anti-BNP required to detect rBNP was 50ng/mL (Fig 3.7a). Moreover, purified anti-BNP was able to detect rBNP as well as BNP from mouse heart samples (Fig 3.7b and Fig 3.10b).

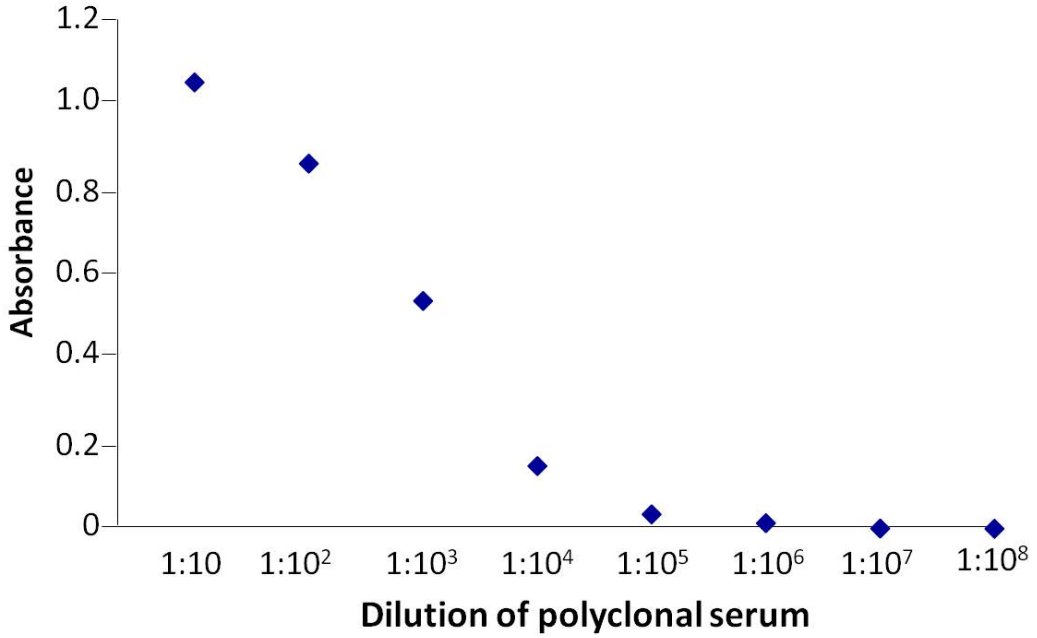

Figure 3.6 Antibody titer for polyclonal serum. Antibody titer was performed using serial dilutions of the polyclonal serum obtained from terminal bleed. The polyclonal serum was able to detect the rBNP at $1:10^4$ dilutions.

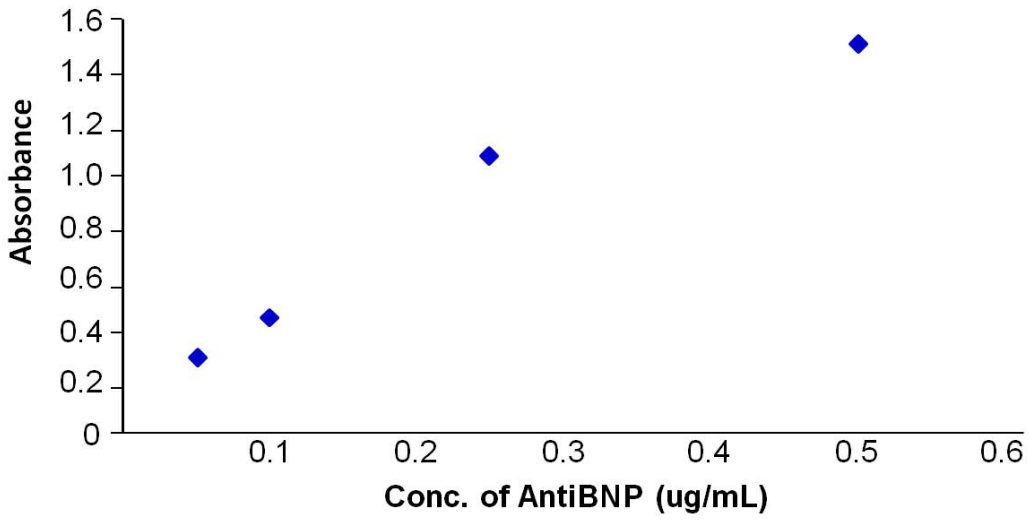

Figure 3.7a Antibody titer for purified anti-BNP. Antibody titer was performed using the purified antiBNP. The purified polyclonal anti-BNP was able to detect the rBNP when used at 50 ng/mL concentration.

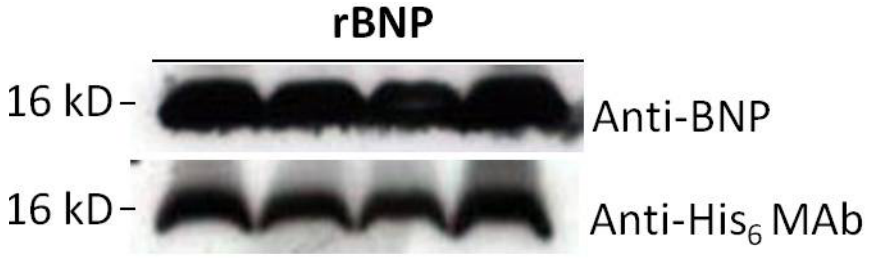

Figure 3.7b Functional test of Anti-BNP. Anti-BNP was able to detect rBNP.

3.3.6 rBNP induced cardioprotection

Recent evidence suggests protective role of BNP against ischemia reperfusion injury.[26-28] To examine the effect of rBNP, first we perfused WT mouse hearts with exogenous rBNP (10nM) to study the effect of our recombinant protein on postischemic contractile function. Hearts treated with exogenous rBNP showed a significant increase in postischemic functional recovery compared to vehicle treated control hearts (62% vs. 23%, respectively, $p<0.05$) (Fig 3.8). Perfusion with the natriuretic peptide receptor-A (NPR-A) antagonist (A71915, 50 nM) attenuated the improved postischemic functional recovery in WT hearts perfused with rBNP (Fig 3.8). These data confirms the previous findings that BNP protects the heart against ischemia reperfusion injury and the effects are mediated through NPR-A. To further assess the cardioprotective effects of rBNP, we analyzed the development of infarct following 2 h reperfusion in rBNP treated hearts and vehicle control hearts. Significant decrease in infarct size was observed in the hearts treated with 10nM rBNP compared to vehicle controls (Fig 3.9).

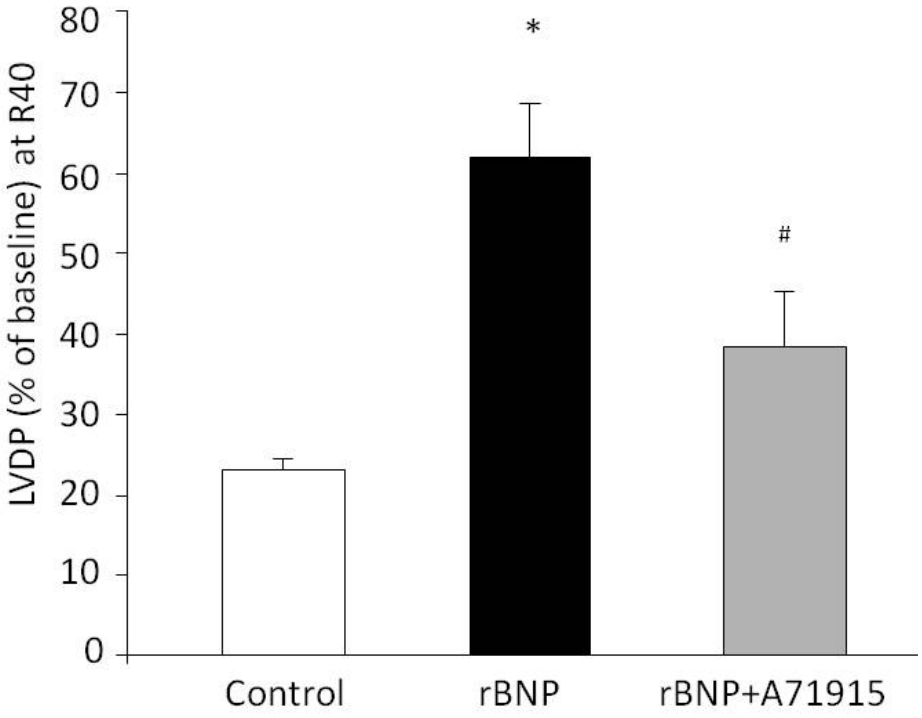

Figure 3.8 rBNP induced cardioprotection. LVDP recovery at 40min of reperfusion of WT hearts treated with rBNP (10nM) or rBNP with A71915 (50nM). Values represent mean ± SEM; n=5-9 per group; *, $p<0.05$ vs. control; #, $p<0.05$ vs. rBNP treated.

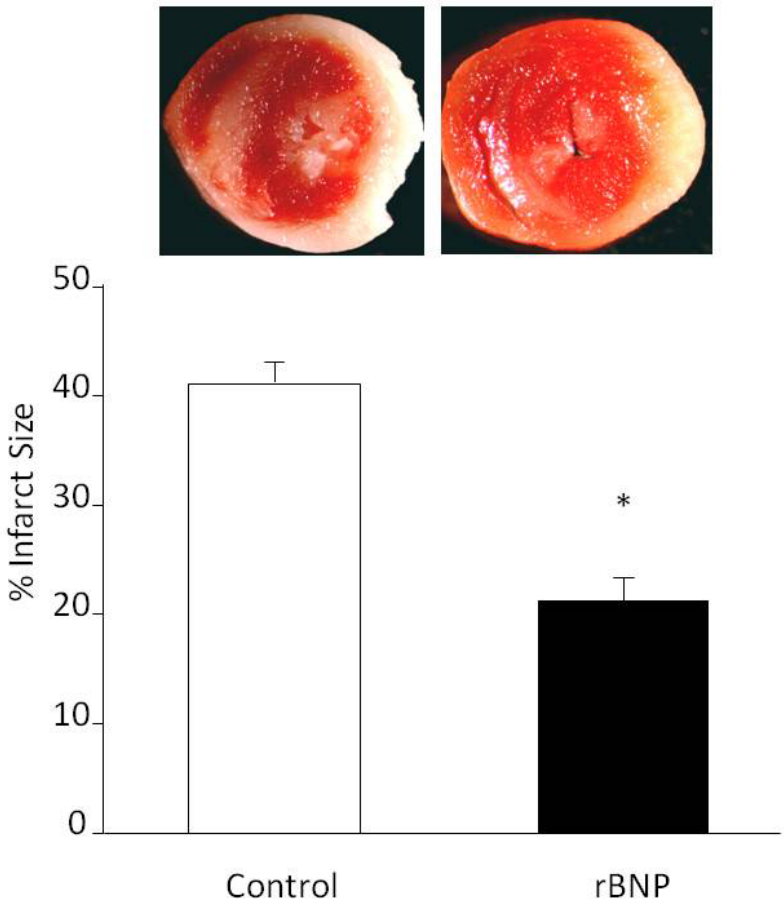

Figure 3.9: Infarct size analysis. Representative images of TTC staining and quantification of infarct size in rBNP and vehicle control hearts. Surviving tissue stained red with TTC and infracted white tissue. Values represent mean±SEM; n=4; *,$p<0.05$ vs. control.

3.3.7 Increased BNP expression in sEH null mouse hearts

As demonstrated earlier, targeted deletion of sEH results in increased Nppb mRNA expression following ischemia reperfusion. Further, we analyzed the isolated perfused hearts for BNP protein expression using the polyclonal anti-BNP antibody. While immunblotting with the anti-mouse BNP antibody demonstrated no significant differences in BNP protein expression following 20 min aerobic perfusion (Fig 3.10a), a significant increase in postischemic BNP protein expression was observed in sEH null hearts compared to WT hearts (Fig 3.10a). In addition, significantly higher expression of BNP was observed in postischemic WT hearts perfused with exogenous 11,12-EET (Fig 3.10b). Densitometry analysis revealed that BNP expression in sEH null mice hearts was four times higher than in WT hearts while exogenous 11,12-EET treated WT hearts had two times higher BNP expression than vehicle treated WT hearts (Fig 3.10b). EET-mediated increased expression was attenuated by co-treatment with 14,15-EEZE (Fig 3.11).

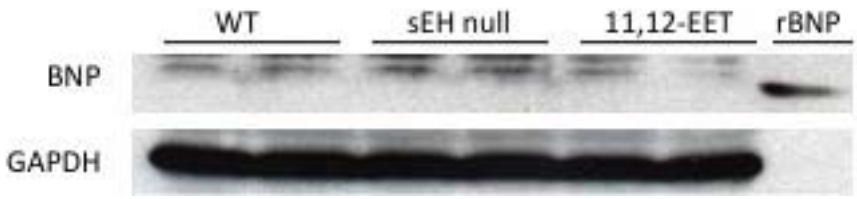

Figure 3.10a BNP expression in preischemic heart samples. Representative immunoblots showing expression of BNP in WT, sEH null and 11,12-EET (1μM) treated hearts during aerobic baseline.

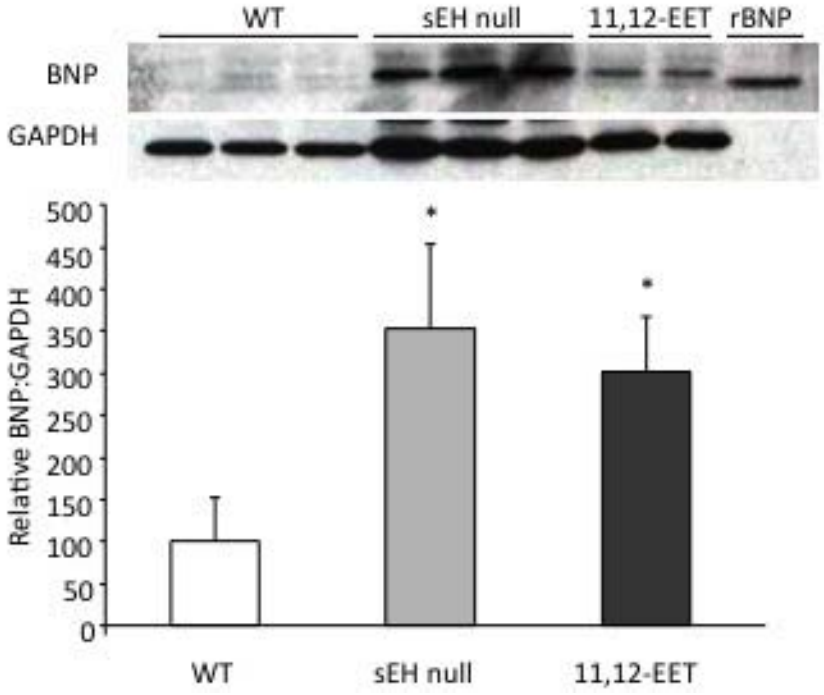

Figure 3.10b BNP expression in postischemic heart samples. Representative immunoblots and densitometry showing relative abundance of BNP to GAPDH in WT, sEH null and 11,12-EET (1μM) treated hearts at 40min reperfusion following global ischemia. Values represent mean ± SEM; n=6 per group; *, $p<0.05$ vs. WT.

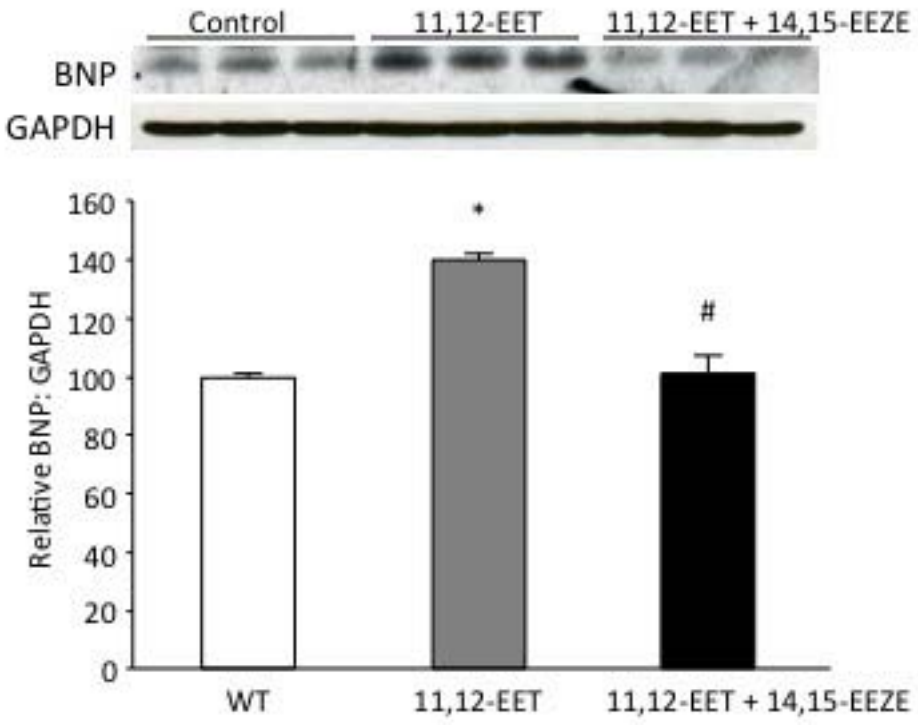

Figure 3.11 BNP expression in EET-perfused heart samples. Immunoblot and densitometry demonstrating BNP expression in vehicle control, 11,12-EET (1μM) or 11,12-EET (1μM) and 14,15-EEZE (10μM) treated hearts. Values represent mean ± SEM; n=3 per group; *, $p<0.05$ vs. Vehicle control; #, $p<0.05$ vs. 11,12-EET treated.

3.3.8 NPR-A signaling in EET and rBNP postischemic contractile function

Consistent with our previous observations[14], sEH null and WT mice had normal baseline contractile function as measured by LVDP (Table 1). Furthermore, sEH null hearts had improved postischemic LVDP recovery following 30min of global ischemia and 40min reperfusion compared to WT hearts (35.6% vs.17.2%, respectively, $p<0.05$) (Fig 3.12; Table 3.1). Likewise, treatment of WT hearts with 11,12-EET improved postischemic LVDP (Fig 3.13; Table 3.2).

Recent evidence suggests a role for BNP in recovery of postischemic cardiac function.[19, 27, 29] The increased BNP expression in sEH null and 11,12-EET treated hearts suggested the hypothesis that BNP may be a mediator of the cardioprotective effects of EETs. To examine the role of BNP, first we perfused WT mouse hearts with exogenous rBNP (10nM) to study the effect of our recombinant protein on postischemic contractile function. Hearts treated with exogenous rBNP did not demonstrate significant changes to baseline LVDP; however, these BNP treated hearts showed a significant increase in postischemic functional recovery compared to vehicle treated control hearts (62% vs. 23%, respectively, $p<0.05$) (Fig 3.13, Table 3.2). Perfusion with the natriuretic peptide receptor-A (NPR-A) antagonist A71915 attenuated the improved postischemic functional recovery in WT hearts perfused with rBNP (Fig 3.13; Table 3.2). To further assess the role of BNP in EET-

mediated cardioprotection, WT hearts were perfused with 11,12-EET in the presence or absence of A71915. Perfusion with A71915 during ischemia and reperfusion significantly inhibited, but did not completely reverse, the improved postischemic recovery observed in 11,12-EET-treated hearts (Fig 3.13; Table 3.2). Moreover, a significant reduction in postischemic functional recovery of sEH null hearts was observed when perfused with A71915 (Fig 3.12). In contrast, there was no effect of A71915 treatment on WT hearts (Fig 3.12). Together, these data suggest that 11,12-EET-mediated cardioprotection dependent upon the NPR-A receptor.

To further assess downstream targets of the NPR-A cascade, we examined the expression of p^{Ser729}PKCε in mitochondrial and cytosolic fractions. In 11,12-EET perfused hearts, significant increases in the phosphorylation of PKCε were observed in mitochondrial fraction (Fig 3.14) while a clear trend towards increase in cytosolic fractions were observed at 40min of reperfusion (Fig 3.17). Consistent with 11,12-EET treated hearts, significant increase in p^{Ser729}PKCε expression in mitochondrial fraction of sEH null hearts was observed compared to WT hearts (Fig 3.15). Similar results were obtained from rBNP perfused hearts with significant increases in mitochondrial and cytosolic p^{Ser729}PKCε levels (Fig 3.16 and Fig 3.18). Evidence suggests that PKCε-mediated cardioprotection involves increased phosphorylation and subsequent inactivation of GSK3β.[13] A significant decrease in mitochondrial total

GSK3β expression was observed in 11,12-EET and rBNP treated hearts, as compared to vehicle treated controls at R40 (Fig 3.19). Conversely, higher expression levels of phosphorylated GSK3β and total GSK3β were observed in cytosolic fractions from hearts perfused with 11,12-EET or rBNP (Fig 3.21). Similar effects on mitochondrial and cytosolic GSK3β total were observed in sEH null hearts compared to WT hearts (Fig 3.20 and Fig 3.22). Together these data indicate that 11,12-EET and BNP trigger phosphorylation of PKCε and this event is associated with phosphorylation and inactivation of GSK3β.

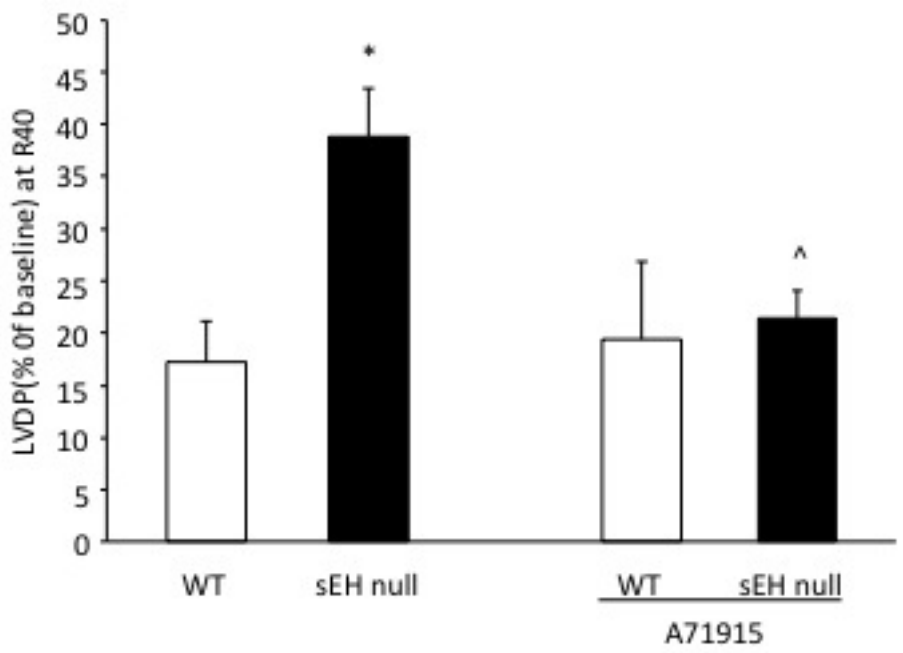

Figure 3.12 Role of NPRA in postischemic functional recovery of sEH null mouse hearts. LVDP recovery at 40min of reperfusion in sEH or WT hearts treated with A71915 (100nM) or vehicle. Values represent mean ± SEM; n=4-9 per group; *, $p<0.05$ vs. WT; ^, $p<0.05$ vs. vehicle control.

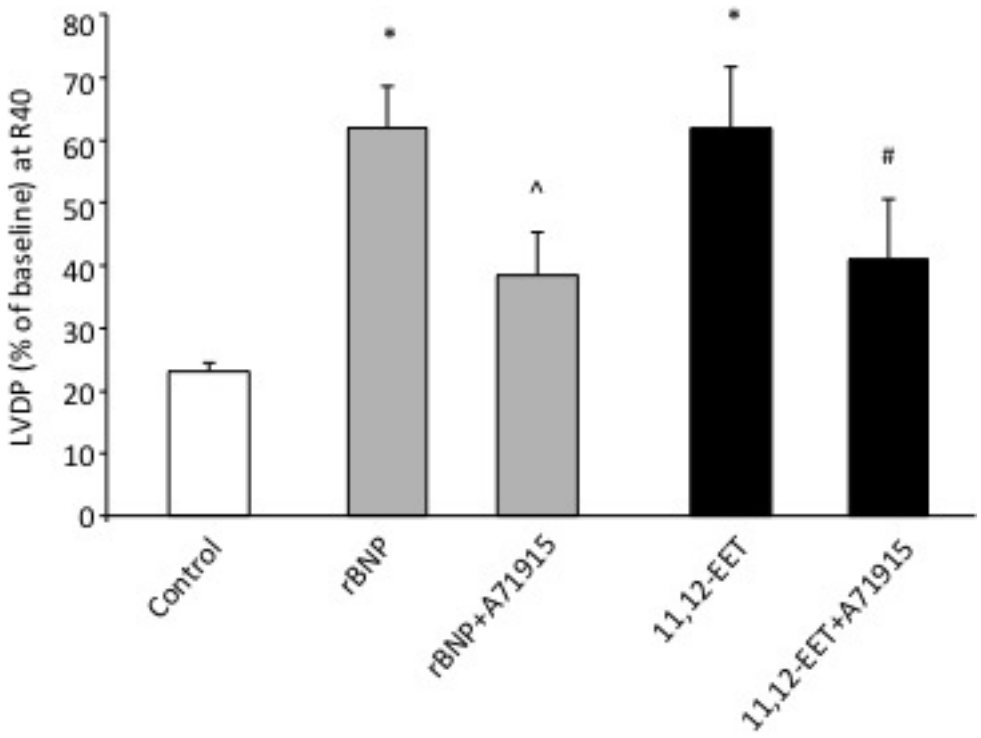

Figure 3.13 Role of NPRA in postischemic functional recovery of EET-perfused WT mouse hearts. LVDP recovery at 40min of reperfusion as percentage of baseline. Hearts were perfused with exogenous rBNP (10nM) or 11,12-EET (1μM) in presence or absence of NPR-A antagonist (A71915) (50nM). Values represent mean ± SEM; n=5-9 per group; *, $p<0.05$ vs. vehicle control; ^, $p<0.05$ vs. treated with rBNP; #, $p<0.05$ vs. treated with 11,12-EET.

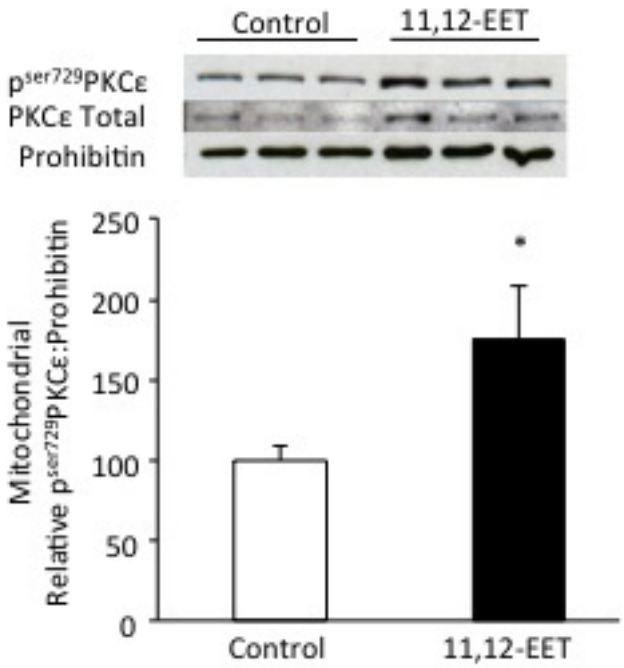

Figure 3.14 PKCε expression in mitochondrial fraction in 11,12-EET-perfused hearts. Representative immunoblot and densitometry showing p^{ser729}PKCε and total PKCε in mitochondrial fractions of 11,12-EET (1μM) or vehicle treated WT mouse hearts. Values represent mean ± SEM; n=6 per group; *, $p<0.05$ vs. vehicle control.

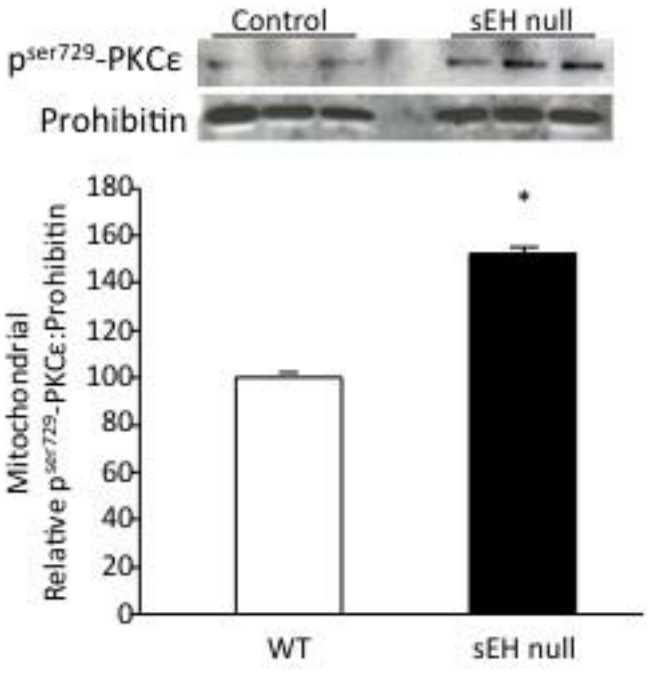

Figure 3.15 PKCε expression in mitochondrial frction in sEH null mouse hearts. Representative immunoblot and densitometry demonstrating p^{ser729}PKCε and total PKCε in mitochondrial fractions of sEH null or WT mouse hearts. Values represent mean ± SEM; n=4 per group; *, $p<0.05$ vs. Vehicle control.

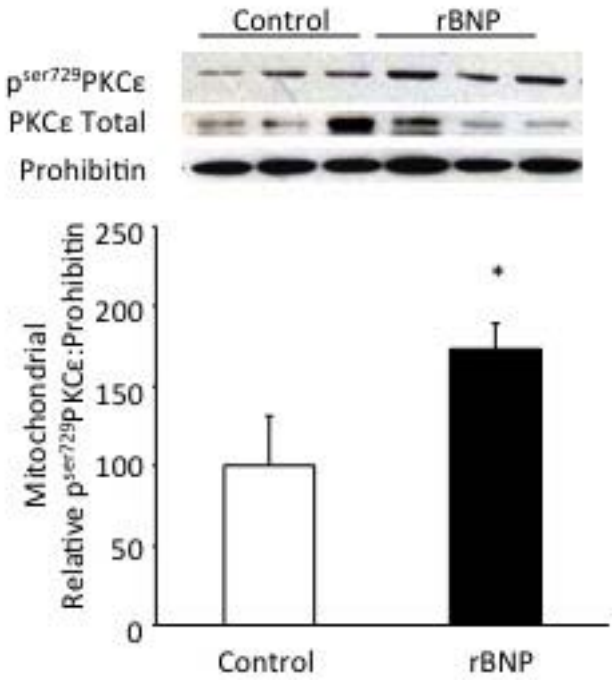

Figure 3.16 PKCε expression in mitochondrial fraction in rBNP-perfused hearts. Representative immunoblot and densitometry demonstrating p^{ser729}PKCε and total PKCε in mitochondrial fractions of rBNP (10nM) or vehicle treated WT mouse hearts. Values represent mean ± SEM; n=5 per group; *, $p<0.05$ vs. Vehicle control.

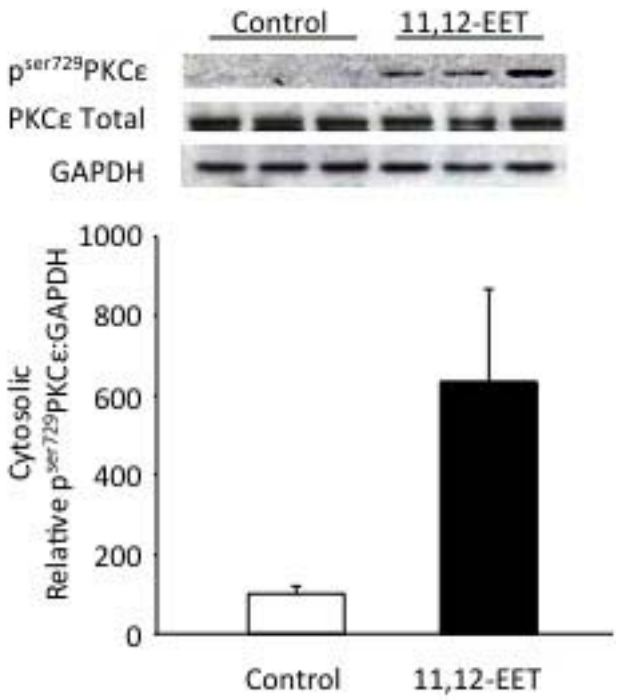

Figure 3.17 PKCε expression in cytosol fraction in 11,12-EET-perfused hearts. Representative immunoblot and densitometry of cytosolic fraction of 11,12-EET (1μM) or vehicle treated C57BL6 mice hearts showing p^{ser729}PKCε and total PKCε expression. Values represent mean ± SEM; n=6 per group; *, $p<0.05$ vs. vehicle control.

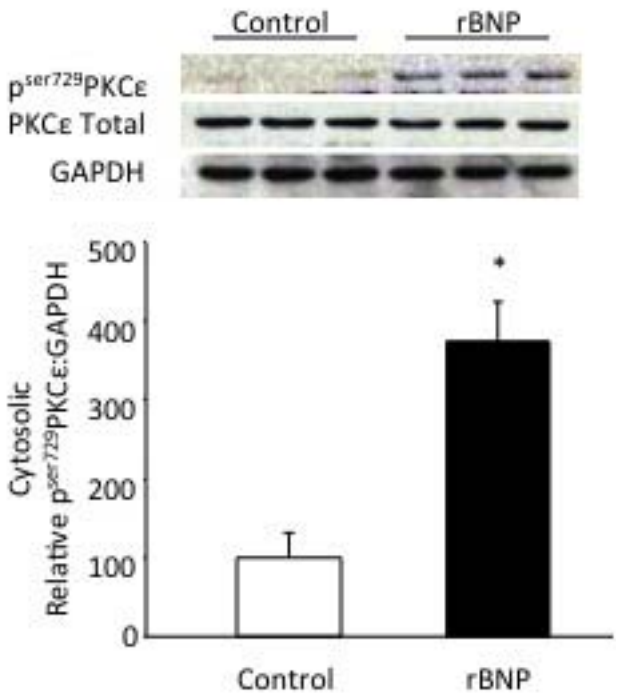

Figure 3.18 PKCε expression in cytosol fraction in 11,12-EET-perfused hearts. Representative immunoblot and densitometry of cytosolic fraction of rBNP (10nM) or vehicle treated WT mouse hearts. p^{ser729}PKCε and total PKCε expression. Values represent mean ± SEM; n=5 per group; *, $p<0.05$ vs. Vehicle control.

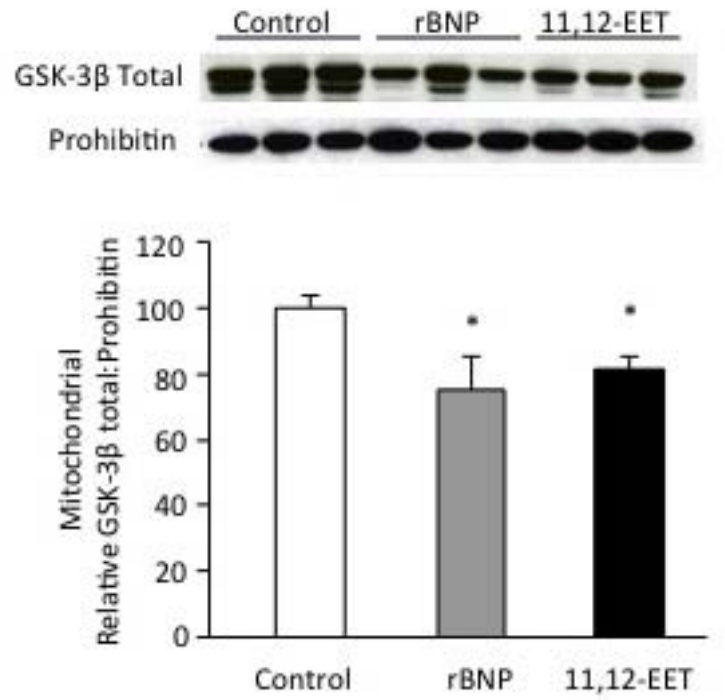

Figure 3.19 Mitochondrial GSK3β expression in ischemic reperfused mouse hearts. Representative immunoblot and densitometry of total GSK3β in mitochondrial fractions of 11,12-EET (1μM), rBNP (10nM) or vehicle treated hearts. Values represent mean±SEM; n=3 per group; *, $p<0.05$ vs. vehicle control.

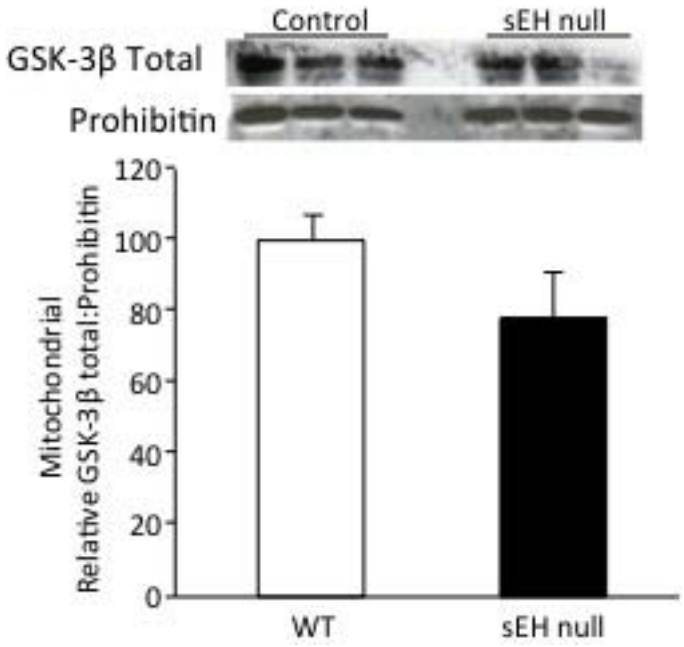

Figure 3.20 Mitochondrial GSK3β expression in ischemic reperfused mouse hearts. Representative immunoblot and densitometry of total GSK3β in mitochondrial fractions of sEH null or WT hearts. Values represent mean±SEM; n=4 per group.

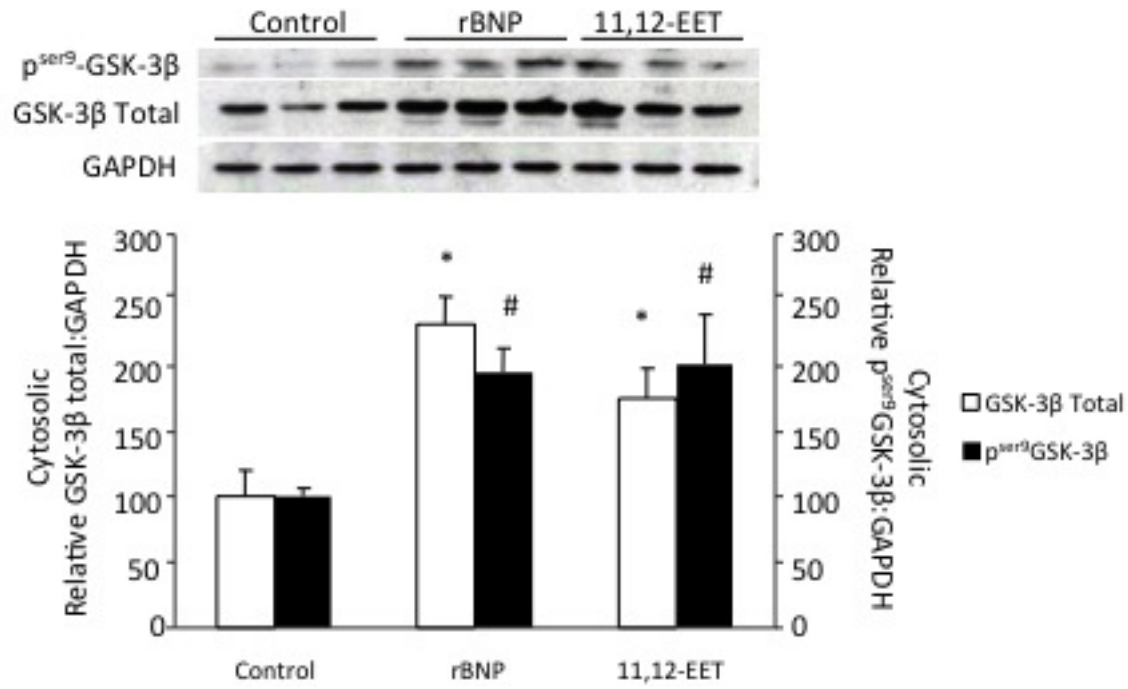

Figure 3.21 Cytosolic GSK3β expression in ischemic reperfused mouse hearts. Representative immunoblot for cytosolic expression of p^{ser9}GSK3β and total GSK3β in 11,12-EET (1μM), rBNP (10nM) or vehicle treated hearts. Values represent mean±SEM; n=3 per group; *, $p<0.05$ total GSK3β, treated vs. vehicle control, #, $p<0.05$ p^{Ser9}GSK3β, treated vs. vehicle control.

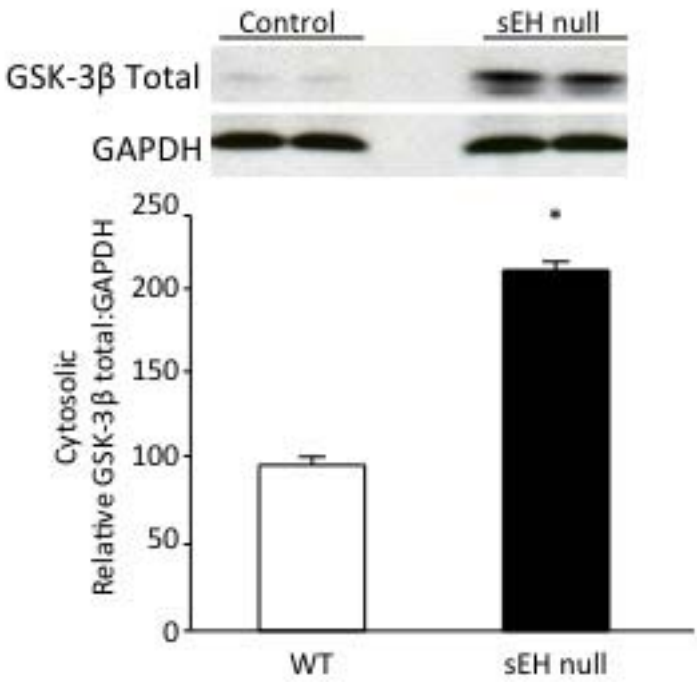

Figure 3.22 Cytosolic GSK3β expression in postischemic sEH null mouse hearts. Representative immunoblot for cytosolic expression of total GSK3β in sEH null and WT hearts. Values represent mean±SEM; n=4 per group; *, $p<0.05$, vs. WT.

3.3.9 Role of PI3K pathway in EET and BNP mediated cardioprotection

As previously documented, EET mediated cardioprotection involves activation of a PI3K pathway while BNP causes activation of a NPR-A/cGMP/PKG pathway.[14, 19, 29] Partial inhibition of EET-mediated cardioprotection by A71915 (Fig 3.13) suggests that EETs may work through multiple pathways. Therefore we investigated the contribution of PI3K and NPR-A in the EET-BNP-mediated cardioprotection. Hearts from WT mice were perfused with 11,12-EET or rBNP in the presence or absence of the PI3K inhibitor wortmannin. Postischemic functional recovery was significantly inhibited in hearts co-perfused with 11,12-EET and wortmannin but did not return to vehicle control levels (Fig 3.23). Interestingly, wortmannin also resulted in a partial inhibition of postischemic functional recovery in hearts co-perfused with rBNP, although these changes were not statistically significant (Fig 3.23). To further assess downstream targets in the PI3K cascade, we examined the phosphorylation status of Akt following ischemia-reperfusion. Expression levels of p^{Ser473}Akt were significantly higher in 11,12-EET, sEH null and rBNP perfused compared to vehicle control hearts at 40min of reperfusion (Fig 3.24, Fig 3.25 and Fig 3.26). Moreover, the ratio of p^{Ser473}Akt to total Akt expression was significantly decreased in WT hearts perfused with both rBNP and A71915 (Fig 3.24). Interestingly, A71915 did not inhibit 11,12-EET mediated increase in p^{Ser473}Akt expression (Fig 3.26). These

data suggest that EET-mediated signaling down the PI3K pathway does not occur solely via the NPR-A receptor

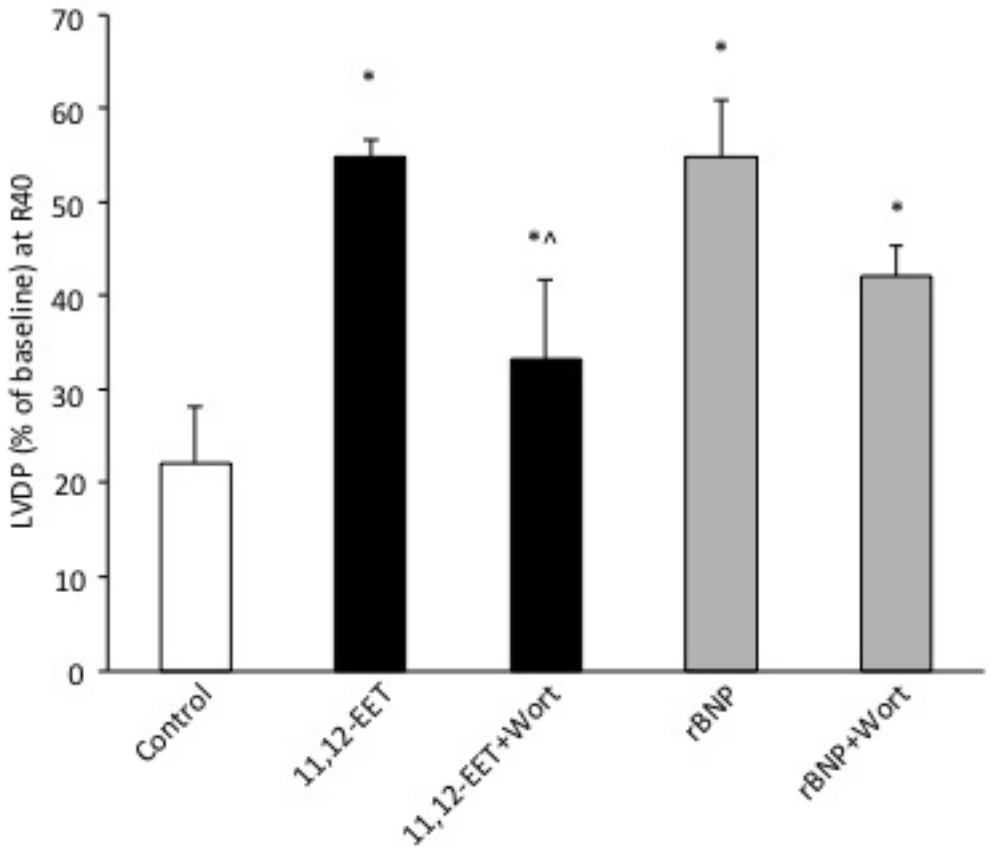

Figure 3.23 PI3K activation in EET and BNP mediated cardioprotection. LVDP recovery at 40min of reperfusion as percentage of baseline. Hearts treated with exogenous rBNP (10nM) or 11,12-EET (1μM) in presence and absence of PI3K antagonist (wortmannin) (200nM). Values represent mean±SEM; n=5-7 per group; *, $p<0.05$ vs. vehicle control; ^, $p<0.05$ vs. treated with 11,12-EET.

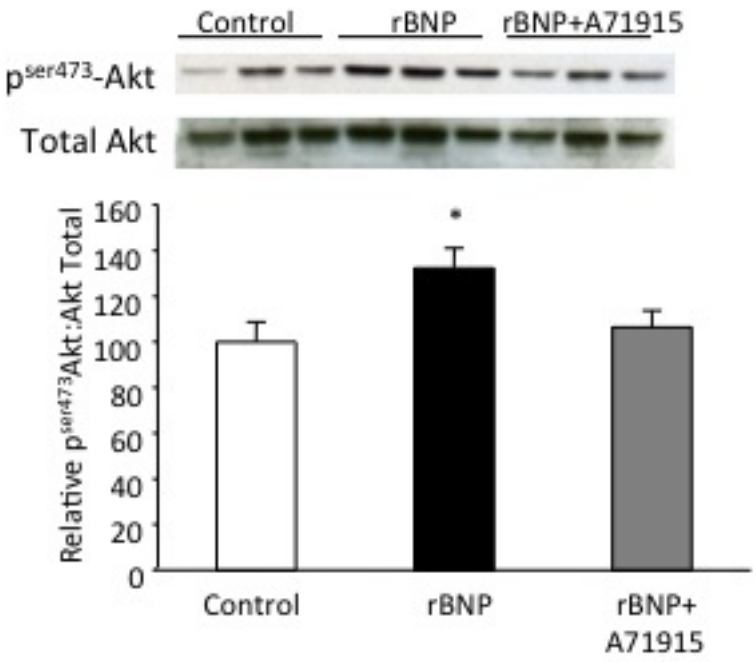

Figure 3.24 Akt activation in BNP mediated cardioprotection. Representative immunoblot and densitometry demonstrating expression of p^{ser473}Akt to total Akt in control, rBNP treated or rBNP and A71915 treated C57BL6 mice hearts. Values represent mean±SEM; n=3 per group; *, $p<0.05$ vs. vehicle control.

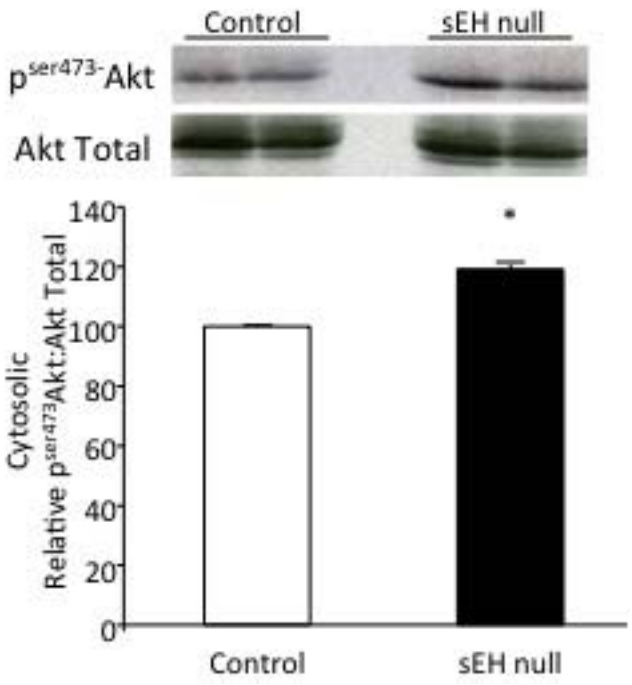

Figure 3.25 Akt activation in postischemic sEH null mouse hearts. Immunoblot and densitometry demonstrating expression of p^{ser473}Akt to total Akt expression in sEH null or WT hearts. Values represent mean±SEM; n=4 per group; *, $p<0.05$ vs. vehicle control.

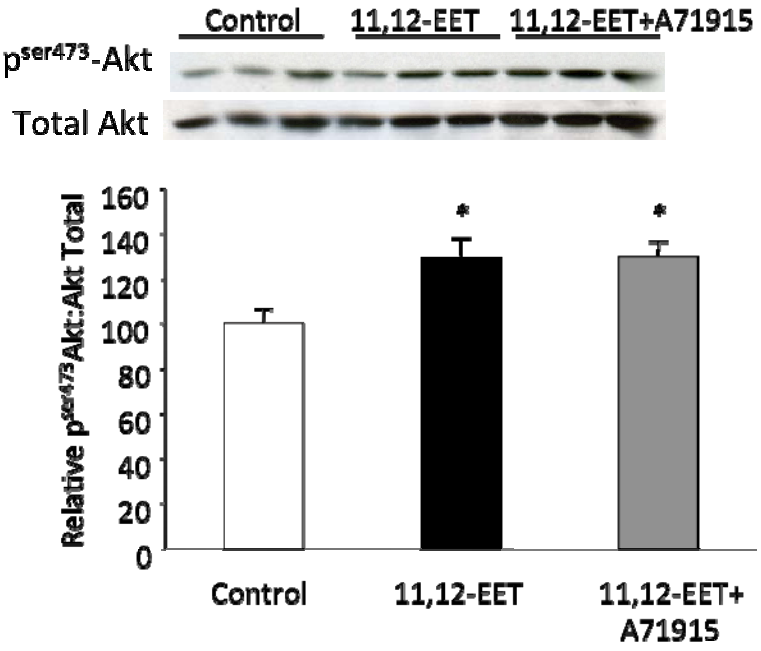

Figure 3.26 Akt activation in EET mediated cardioprotection. Immunoblot and densitometry demonstrating expression of p^{ser473}Akt to total Akt expression in control, rBNP treated or rBNP and A71915 treated hearts. Values represent mean±SEM; n=6 per group; *, $p<0.05$ vs. vehicle control.

Table 3.1 Cardiac parameters in sEH null and WT mice

	WT (*n*=6)	sEH null (*n*=8)	WT+ A71915 (n=3)	sEH null + A71915 (n=4)
Isolated Perfused Heart - Preischemic				
LVDP (cmH_2O) (Baseline)	131.4±18	129±10.8	145.0±15.0	122.3±9.2
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (Baseline)	3830±515	3389±575	4289±628	4086±669
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (Baseline)	-3234±372	-2770±506	-3351±376	-3324±428
HR, perfused (beats/min) (Baseline)	319±22	290±29	280±31	345±9
Isolated Perfused Heart - Postischemic				
LVDP (cmH_2O) (R40)	21.1±4.0	50.9±7.8*	30.3±13.8	25.8±2.9$^{\#}$
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (R40)	658±119	1484±139*	631±155	748±68$^{\#}$
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (R40)	-540±100	-1369±185*	-506±103	-623±59$^{\#}$
HR, perfused (beats/min) (R40)	252±33	248±23	258±26	336±13$^{\#}$

Hemodynamic parameters were measured in isolated-perfused hearts. Values represent mean±SEM,

* $p<0.05$ vs WT; $^{\#}$ $p<0.05$ vs. sEH null. LVDP, left ventricular pressure, HR, heart rate.

Table 3.2: Cardiac parameters in control and rBNP or 11,12-EET treated mice

	Control (*n*=8)	11,12-EET (*n*=6)	rBNP (*n*=5)	11,12-EET + A71915 (*n*=4)	rBNP + A71915 (*n*=5)	11,12-EET + Wortmannin (*n*=5)	rBNP + Wortmannin (*n*=5)
Isolated Perfused Heart – Preischemic (Baseline)							
LVDP (cmH_2O)	108.2±10.3	116.6±8.7	102.4±9.1	113.6±5.6	105.5±7.4	137.8±22.2	126.4±16.0
Rate of contraction, dP/dt_{max} (cmH_2O/msec)	3167±397	3025±190	2649±156	3249±167	2890±251	4285±668	4133±589
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec)	-2624±247	-2608±192	-2198±176	-2589±203	-2454±192	-3301±507	-3111±403
HR, perfused (beats/min)	302±18	359±8	315±31	329±19	346±13	277±33	330±14
Isolated Perfused Heart – Postischemic (R40)							
LVDP (cmH_2O)	25.4±3.2	74.6±8.7*	61.5±3.1*	42.2±9.4$^{\#}$	29.6±6.9^	42.2±9.4$^{\#}$	54.5±7.9*
Rate of contraction, dP/dt_{max} (cmH_2O/msec)	804±107	2291±276*	1787±117*	1167±52$^{\#}$	858±123^	1439±246$^{\#}$	1539±191*
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec)	-704±91	-1979±225*	-1522±112*	-980±50$^{\#}$	-781±109^	-1234±221	-1294±123*
HR, perfused (beats/min)	313±18	328±11	304±18	330±21	324±20	382±61	291±29

Hemodynamic parameters were measured in isolated-perfused hearts. Values represent mean±SEM, * $p<0.05$ vs Vehicle control; $^{\#}$ $p<0.05$ vs. 11,12-EET treated; ^ $p<0.05$ vs. rBNP treated. LVDP, left ventricular pressure, HR, heart rat.

3.4 Discussion

Since the first report of the cardioprotective effects CYP epoxygenase metabolites of arachidonic acid against ischemia reperfusion injury by Wu et al in 1997, there has been considerable interest in investigating this novel protective mechanism.[7, 14, 23] However; important questions remain unanswered regarding the molecular and cellular mechanisms responsible for this protection. Evidence suggests the cardioprotective signaling of EETs involves activation of PI3K/Akt pathway which further prevents ischemic injury by reducing mitochondrial damage through activation of mitoK_{ATP} channels.[14] Herein, we demonstrate for the first time, that targeted disruption of the *Ephx2* gene and treatment with exogenous 11,12-EET leads to increased BNP expression following ischemia-reperfusion resulting in improved functional recovery. Moreover, our data suggest a critical role of BNP and GSK3β in mediating the cardioprotective effect of EETs. Thus, EET-mediated cardioprotection involves two pathways, PI3K/Akt and BNP-PKCε-mediated signaling, which converge on the mitochondria. Taken together, these data suggest that EETs have important intracellular protective effects in the ischemic heart that limit functional damage attributed to ischemia-reperfusion injury.

BNP is a cardiac hormone predominantly found in ventricles of heart. Considered an immediate-early gene, it can be both rapidly synthesized and released within 1 hr in response to stimuli like stretch,

volume and pressure overload and ischemia.[30] Basal expression levels of Nppb mRNA were found to be similar in naïve sEH null and WT hearts, indicating targeted disruption of *Ephx2* gene does not alter BNP under normal conditions. Studies in isolated rat hearts demonstrate that increased Nppb mRNA expression was observed during aerobic perfusion, reflecting a stretch induced activation.[31-32] We observed increased Nppb mRNA levels during aerobic perfusion which was slightly higher in sEH null hearts compared to WT hearts. This early increased expression did not translate into elevated BNP protein levels. However, the significant increases in Nppb mRNA levels observed in sEH null hearts following ischemia reperfusion correlated with large increases in BNP protein levels in sEH null hearts and WT mice perfused with EETs. Consistent with our previous results from sEH null mice, cardiac effects of sEH disruption occurred only after ischemia, presumably due to enhanced release of EETs from phospholipid stores.[14] A role for EETs in the induction of BNP is suggested by our observation that the putative EET receptor antagonist 14, 15-EEZE attenuates the increase in Nppb mRNA in sEH null mice.

Apart from mechanical stimulus, BNP gene expression can be induced in response to various factors such as endothelin-1, β-adrenergic stimulation and proinflammatory cytokines, like interleukin-1β.[33] Interleukin-1β induces BNP gene expression, partially, through a Ras, Rac and p38 kinase pathway, where p38 kinase acts on the MCAT element at

position-97 of human BNP gene promoter.[33-34] Alternatively, stabilization of Nppb mRNA via an MAPK and PKC dependent mechanism results in increased levels as demonstrated in neonatal rat cardiomyocytes [35]. While the mechanism for EET-mediated activation of BNP is not known, the observation that LY294002 did not affect increased Nppb mRNA in sEH null hearts suggests that the PI3K pathway is not involved. Our results demonstrate both stretch and ischemia-reperfusion injury increase Nppb mRNA expression in WT hearts which was markedly enhanced in sEH null hearts. Importantly, increased Nppb mRNA correlated with elevated BNP protein expression in hearts of both sEH null and WT mice perfused with EETs following ischemia-reperfusion. These results provide further evidence for the cardioprotective effects EETs and suggest they act as a novel regulator of BNP release.

Following ischemia rapid gene expression and *de novo* peptide synthesis occurs which results in increased plasma levels of BNP in acute myocardial infarction patients.[30] BNP plasma concentrations are biomarkers for heart disease providing a valuable diagnostic tool for cardiac patients.[36] However, the role of BNP in the pathophysiology of ischemia-reperfusion injury and whether the heart is a target remains to be elucidated. BNP can exert autocrine or paracrine effects by binding with natriuretic peptide receptor type- A (NPR-A), which activates particulate guanylyl cyclase (pGC), thereby increasing formation of cGMP leading to activation of PKG.[37] PKG can then activate PKC, particularly PKCε,

which will translocate to mitochondria interacting with various mitochondrial membrane proteins, such as mitoK_{ATP} channels.[13, 38] Translocation of PKCε to mitochondria and activation of mitoK_{ATP} channels are believed to protect the myocardium from ischemia-reperfusion injury, with a net effect of protecting mitochondrial function.[13, 39] D'Souza et al demonstrated that perfusion of rat hearts with endogenous BNP induces protection against reperfusion injury involving pGC and activation of K_{ATP} channels.[27] We established that EET-mediated cardioprotection in CYP2J2 transgenic and sEH null mice involve activation K^+ channels; however, the exact intracellular signals remain unknown.[14, 23] In the present study, we observed increased levels of PKCε in mitochondrial fractions of hearts perfused 11,12-EET or rBNP, which correlated with improved postischemic functional recovery. Furthermore, administration of NPR-A antagonist, A71915, attenuated the improved postischemic recovery of sEH null hearts and hearts perfused with rBNP peptide but only partial inhibition was observed with exogenous EETs. Taken together, our experiments suggest that EET-mediated cardioprotection, at least in part, involves NPR-A activation of intracellular pathways that target the mitochondria.

GSK3β is a constitutively active protein which is an important regulator of cellular function and a key enzyme in response to myocardial ischemia-reperfusion injury.[13] Inhibition of GSK3β, either by increased phosphorylation or pharmacological inactivation, is known to reduce cell

death caused by ischemia-reperfusion injury. Increased expression of GSK3β in mitochondria following ischemia-reperfusion has been reported in cardiomyocytes and isolated hearts experiments.[40] The significance and mechanism of GSK3β translocation has not been determined but evidence suggests a role for PI3K and PKC kinases.[40] In addition, the specific effect of increased mitochondrial GSK3β is not completely known but potentially involves modulation of function. Juhaszova et al hypothesized that cardioprotective signaling pathways converge onto GSK3β resulting in prevention of mitochondrial permeability transition (mPTP).[13] Recent work by Das et al report that inhibition of GSK activity induces dephosphorylation of VDAC, which subsequently preserves ATP levels, prevents calcium overloading and oxidant stress independent of mPTP opening.[41] Phosphorylation of GSK3β by upstream kinases like Akt and PKC inactivate GSK3β preventing myocardial damage from ischemia-reperfusion injury.[42] Previously we demonstrated that cardioprotection in sEH null mice involves activation of PI3K followed by phosphorylation of GSK3β.[14] Interestingly, our current results demonstrate a significant reduction in active GSK3β in mitochondrial fractions of both 11,12-EET and rBNP treated hearts compared controls. In addition, we observe increased levels of active Akt (p^{ser473}Akt) and inactive GSK3β (p^{ser9}GSK3β) in cytosolic fractions, we predict that EETs and BNP either prevent the translocation of GSK3β to the mitochondria or enhance the translocation from the mitochondria. In addition, our data

suggest that BNP-mediated effects on GSK3β do not involve PI3K/Akt pathway but activation of Akt via the NPR-A receptor. In contrast, EET-mediated events involve both PI3K/Akt and enhanced release of BNP. These data are consistent with evidence suggesting that inhibition of GSK3β is a key component in integrating protective stimuli with mitochondria.[7, 13, 16] However, the relative contribution of each pathway (PI3K/Akt or NPR-A) to the regulation of GSK3β is unknown and requires further investigation.

3.5 Conclusion

We propose that enhanced postischemic functional recovery attributed to EETs is mediated through two different signaling pathways - PI3K/Akt and BNP/NPR-A signaling. Figure 3.27 shows a schematic of the proposed mechanisms whereby EETs can trigger the release of BNP to act in an autocrine manner or activate the PI3K pathway. Together the data in the current study provide further evidence for the beneficial effects of EETs and suggest a novel mechanism for BNP in cardioprotection.

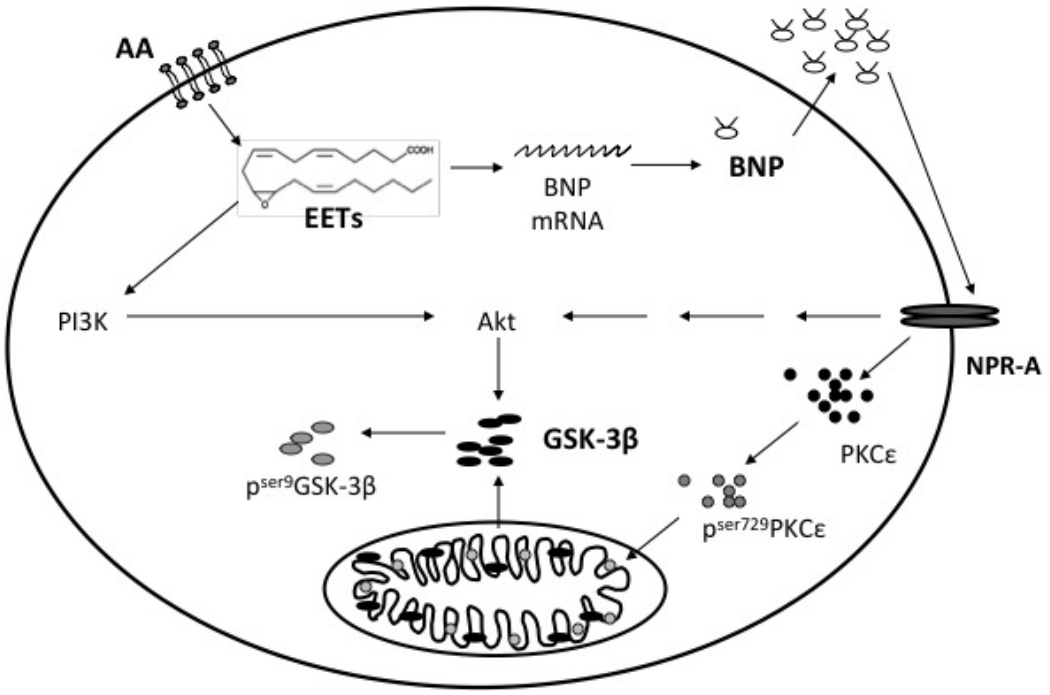

Figure 3.27 Schematic diagram showing pathways of EET mediated improved left ventricular function. We propose that the enhanced postischemic functional recovery attributed to EETs is mediated through two different signaling pathways - PI3K/Akt and BNP/NPR-A signaling. First, EETs can activate the PI3K/Akt pathway which will inactivate GSK-3β. Or second, EETs can activate the BNP/NPR-A pathway, which in turn can either activate Akt independent of PI3K, or activate PKCε.

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Chapter 4

Effect of aging on epoxyeicosatrienoic acid induced improved postischemic recovery of heart contractile function

[4]A version of this chapter has been submitted for publication: Chaudhary KR, Zordoky BNM, Edin ML, El-Kadi AO, Zeldin DC, Seubert JM. Epoxyeicosatrienoic acids improve postischemic functional recovery of heart in aged mice. *Prostaglandins Other Lipid Mediat. April 2012.*

4.1 Introduction

Cytochrome p450 (CYP) epoxygenases, CYP2C and CYP2J subfamilies enzymes, play important role in fatty acids metabolism.[1] In the heart, CYP2J is abundant and predominantly involved in the biosynthesis of epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs) from arachidonic acid.[2] EETs play important role in cellular signaling and possess numerous biological activities in the cardiovascular system including vasodilatory, anti-inflammatory, anti-fibrotic, natriuretic and anti-apoptotic.[3-4] Modulation of EET levels can occur via reincorporation into phospholipid membranes or by β-oxidation to smaller reactive epoxides;[4-6] however, the predominant pathway occurs through metabolism to inactive vicinal diols compounds by epoxide hydrolases.[4, 6-7] Two major epoxide hydrolases are found in mammalian tissues, microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH or *Ephx2*).[8]

Accumulating evidence indicate that EETs have important functional roles in ischemia-reperfusion injury.[9-13] Studies from our group and others have demonstrated cardioprotective effects of EETs.[13-16] Studies reported that mice with the targeted deletion of the *Ephx2* gene (sEH knockout, sEH null) had enhanced postischemic recovery of left ventricular function, which was mediated by activation of the PI3K pathway and K^+ channels.[10, 14] Inhibition of soluble epoxide hydrolase, using pharmacological inhibitors (sEHi), protects the heart against

ischemia reperfusion injury.[12-13] Cardiomyocyte specific over-expression of CYP2J2 in CYP2J2 Tr mice leads to improved functional recovery and reduced infarct size after ischemia.[9, 17] Moreover, treatment with exogenous EETs has also been demonstrated to be protective against ischemia reperfusion injury.[15, 18] According to current knowledge, the cardioprotective mechanism(s) of EETs suggest involvement of signaling pathways including phosphoinositide 3-kinase (PI3K) – Akt, increased secretion of cardiac hormones, and activation of cardiac ion channels such as ATP-sensitive K^+ channels and BK_{Ca} channels.[9-10, 14-15, 18]

The growing elderly population has significantly increased interest in age-related diseases, particularly related to the heart. Importantly, this population has a higher risk of cardiovascular disease, which is reflected by death rates of approximately 1000 times higher in individuals who are 85–89 years old compared to those of 25–29 years of age. [19-21] The increased death rate can be explained by an increased susceptibility of aged hearts to stress compared to younger counterparts.[22-23] Indeed, aging causes a significant reduction in the heart's ability to tolerate damage stemming from ischemia reperfusion injury. [22-23] Consequences of aging lower the heart's ability to resistance ischemia reperfusion injury as well decrease the effectiveness of cardioprotective strategies.[24] Therefore, it is important to evaluate the effectiveness of cardioprotective strategies in aged animal models.

While the cardioprotective effects of EETs are well studied in young animal models, there is a lack of information about EET-induced cardioprotection in aged animals. Therefore, in the present study, we examined the effect of aging on EET-induced cardioprotection using young and aged; CYP2J2 Tr and sEH null mice. We demonstrate that aging causes loss of EET-mediated cardioprotection in CYP2J2 Tr but not in sEH knockout mice. Moreover, our data suggest the loss of protective effects in aged CYP2J2 Tr mice can be prevented by sEHi. Taken together, these data suggest that EETs can protect the aged mouse hearts against ischemia reperfusion injury and sEH is a better therapeutic target for protecting the heart against ischemia reperfusion injury.

4.2 Material and Methods

4.2.1 Animals

Mice colonies with targeted disruption of the Ephx2 gene (sEH null) and cardiac myocyte-specific over expression of human CYP2J2 (CYP2J2 Tr) backcrossed onto a C57BL6 genetic background for more than 10 generations were maintained at the University of Alberta. C57BL6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ). All experiments used male and female mice aged 2-3 months (young) or 11-13 months (aged) and were treated in accordance with the guidelines of Health Science Laboratory Animal Services (HSLAS), University of Alberta. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.2.2 Isolated heart perfusions

Hearts were perfused in the Langendorff mode as previously published.[9-10] Briefly, hearts were perfused with Krebs-Henseleit buffer for 40min of baseline and subjected to 30min of global no flow ischemia followed by 40min of reperfusion. For some experiments, hearts were perfused with trans-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB; sEH inhibitor, 100 nM), MS-PPOH (CYP epoxygenase inhibitor, 50 μM) (Cayman Chemicals, Ann Arbor, MI, USA). The percentage of left ventricular developed pressure (%LVDP) at 40min

of reperfusion (R40), as compared to baseline LVDP, was taken as a marker for recovery of contractile function. After 40min of reperfusion, hearts were immediately frozen and stored below -80°C.

4.2.3 Tissue homogenization and sub-cellular fractionation

Cytosolic and microsomal fractions were prepared from frozen mouse hearts as described.[25] For preparation of subcellular fractions, tissues were minced and homogenized in homogenization buffer containing (sucrose 250 mM, TrisHCL 10 mM, EDTA 1 mM, sodium orthovanadate 1 mM, sodium flouride 1 mM, aproptinin 10 µL/L, leupeptin 2 µL/L, pepstatin 100 µL/L). The homogenate was centrifuged at 700 × g for 10 min to remove the debris and the supernatant was again centrifuged to 10,000 × g for 20 min at 4 °C. The resulting supernatant (S-9) fraction and pellet were separated and the pellet was used as the mitochondria rich fraction. The supernatant obtained from further centrifugation of the S-9 fraction at 100,000 × g for 1 h at 4 °C was used as the cytosolic fraction and pellet was used as microsomal fraction. The protein concentration of these fractions was determined colorimetrically using a Bio-Rad BCA protein assay kit using bovine serum albumin as a standard.

4.2.4 Epoxide hydrolase activity analysis

Heart cytosol and microsomes (1 mg of protein/mL) were incubated in the incubation buffer (5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer, pH = 7.4) at 37°C in a shaking water

bath (50 rpm). A pre-equilibration period of 5 min was performed. 14,15-EET was added to a final concentration of 50 μM and incubated for 30 min. The reaction was terminated by the addition of 600 μL of ice-cold acetonitrile followed by the internal standard, 4-hydroxybenzophenone. 14,15-EET and 14,15-DHET were extracted by 1 mL of ethyl acetate twice and dried using a speed vacuum (Thermo Fisher Scientific).

Extracted 14,15-EET and 14,15-DHET were analyzed using the LC-ESI-MS (Micromass ZQ 4000 spectrometer; Waters, Milford, MA) method as described previously.[26] Briefly, the mass spectrometer was operated in negative ionization mode with single ion recorder acquisition. The nebulizer gas was acquired from an in-house high-purity nitrogen source. The source temperature was set at 150°C, and the voltages of the capillary and the cone were 3.51 kV and 25 V, respectively. The samples (10 μL) were separated on a reverse-phase C18 column (Kromasil, 250 × 3.2 mm) using a linear gradient mobile phase system with a mobile phase of water-acetonitrile with 0.005% acetic acid at a flow rate of 0.2 mL/min. The mobile phase system started at 60% acetonitrile, linearly increased to 80% acetonitrile in 30 min, increased to 100% acetonitrile in 5 min, and held for 5 min.

4.2.5 Protein phosphatase 2A (PP2A) activity assay

PP2A activity was determined in heart samples with the ‘PP2A Immunoprecipitation Phosphatase Assay Kit’ by Millipore (Billerica, MA, USA) according to manufacturer’s guideline. Briefly, cytosolic fractions

were incubated with 4 µg of anti-PP2Ac antibodies and protein A/G plus agarose at 4°C with continuous shaking 1200 RPM. Immuno-complexes were washed three times with TBS and one time with PP2A buffer (250 mM imidazole, 1 mM EGTA, 0.1% β-mercaptoethanol and 0.5 mg/mL BSA). Phosphatase activity was assayed by suspending the final pellet in 80 µL PP2A buffer containing 750 mM phosphopeptide substrate RRA(pT)VA for 10 minutes at 37°C. 25 µL of supernatant was incubated with 100 µL of the molybdate dye solution. The reaction was carried out at room temperature for 10 min and absorbance was read at 600 nm.

4.2.6 Immunoblot analysis

Protein was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted as previously described.[9] Immunoblots were prepared using cytosolic (50 µg protein) or microsomal (25 µg protein) fractions and probed with antibodies to sEH (Santacruz biotechnology), PP2A (Millipore) cAMP activated protein kinase (AMPK), phospho- AMPK (p^{Thr182}AMPK), CYP2J2, beta-actin and GAPDH (Cell Signaling Technology, Inc., Danvers, MA). GAPDH and prohibitin were used as loading controls for cytosolic and mitochondrial fraction, respectively. 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.

4.2.7 Eicosanoid analysis

Eicosanoid analysis was performed using liquid chromatography, tandem mass spectroscopy (LC/MS/MS) as described previously. [17] Briefly, each minute 1 mL of perfusate was collected from last 20 min of baseline, spiked with 30 ng PGE2-d4, 10,11-DiHN, and 10,11-EpHep (Cayman) as internal standards, mixed with 0.1 vol of 1% acetic acid in 50% methanol, and extracted by serial passage through Oasis HLB C18 3mL columns (Waters, Milford, MA, USA). Liquid chromatography of the sample was performed with an Agilent 1200 Series capillary HPLC (Agilent Technologies, Santa Clara, CA, USA). Negative ion electrospray ionization tandem mass spectrometry was used for detection.

4.2.8 Protein carbonyl assay

Protein carbonyl ELISA was performed to measure protein oxidation or oxidative stress. ELISA was performed using OxiSlect^{TM} protein carbonyl ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA). The assay was performed according to manufacturer's instructions. The frozen (-80° C) heart samples were powdered in liquid nitrogen using mortar and pastel. Proteins from the powdered samples were extracted using the homogenization buffer and centrifuged at 10000 × g for 20 min at 4 °C. Total protein in the supernatant was measured by Bradford assay. All the samples were diluted to 10 µg/mL using PBS. Nuclei acids were removed by incubating the sample with streptomycin sulfate (1%) for 30 min at room temp. Nuclei acid precipitates were removed by centrifuging the

samples at 6000× g for 10 min at 4 °C. 100 µL of the diluted samples were analyzed for protein carbonylation according to manufacturer's protocol.

4.2.9 Statistical analysis

Values expressed as mean ± standard error of mean (SEM). Statistical significance was determined by the unpaired Student's t-test and one-way ANOVA. To determine whether significant difference exists between the groups, Newman-Keuls post-hoc test was performed. Values were considered significant if $p < 0.05$.

4.3 Results

4.3.1 Post-ischemic cardiac function in young and aged CYP2J2 Tr mice

Young and aged CYP2J2 Tr and WT mice had normal baseline contractile function as measured by LVDP (Fig 4.1a). Significant improvement in postischemic contractile function was observed in young CYP2J2 Tr mice compared to age matched WT (43.1% vs. 23.3%, respectively, $p<0.05$) (Fig 4.1a, 4.1b). Consistent with increased postischemic LVDP, we observed significantly improved postischemic contraction and relaxation rate of young CYP2J2 Tr mouse hearts compared to WT hearts. In contrast, hearts from aged CYP2J2 Tr mice did not have improved postischemic functional recovery compared to WT mice (15.4% vs. 14.8%, respectively). In addition, a significant decrease in postischemic contraction and relaxation rate was observed in aged CYP2J2 Tr hearts compared to young CYP2J2 Tr mouse hearts (Fig 4.2a, 4.2b). No difference was observed in heart rate between any groups (Fig 4.2c).

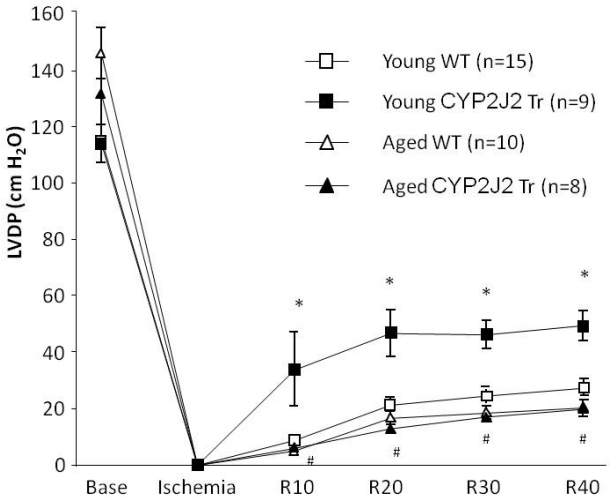

Figure 4.1a LVDP of young and aged CYP2J2 Tr mouse hearts. LVDP at baseline, at the end of ischemia, and at reperfusion (R10, R20, R30, and R40) in young and aged, WT and CYP2J2 Tr hearts. Values represent mean ± SEM; n=8-15 per group; *, $p<0.05$ vs. WT; #, $p<0.05$ vs. young animal of same genotype.

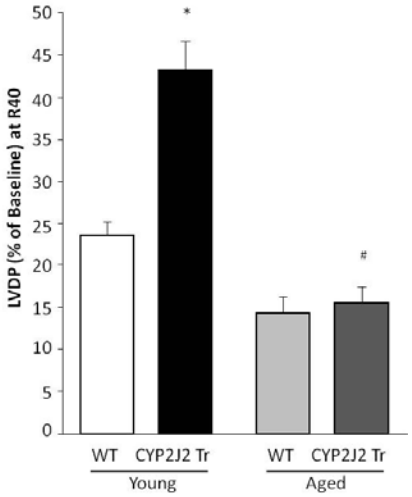

Figure 4.1b LVDP recovery of young and aged CYP2J2 Tr mouse hearts. LVDP recovery at 40min of reperfusion in young and aged, WT and CYP2J2 Tr hearts. Values represent mean ± SEM; n=8-15 per group; *, $p<0.05$ vs. WT; #, $p<0.05$ vs. young animal of same genotype.

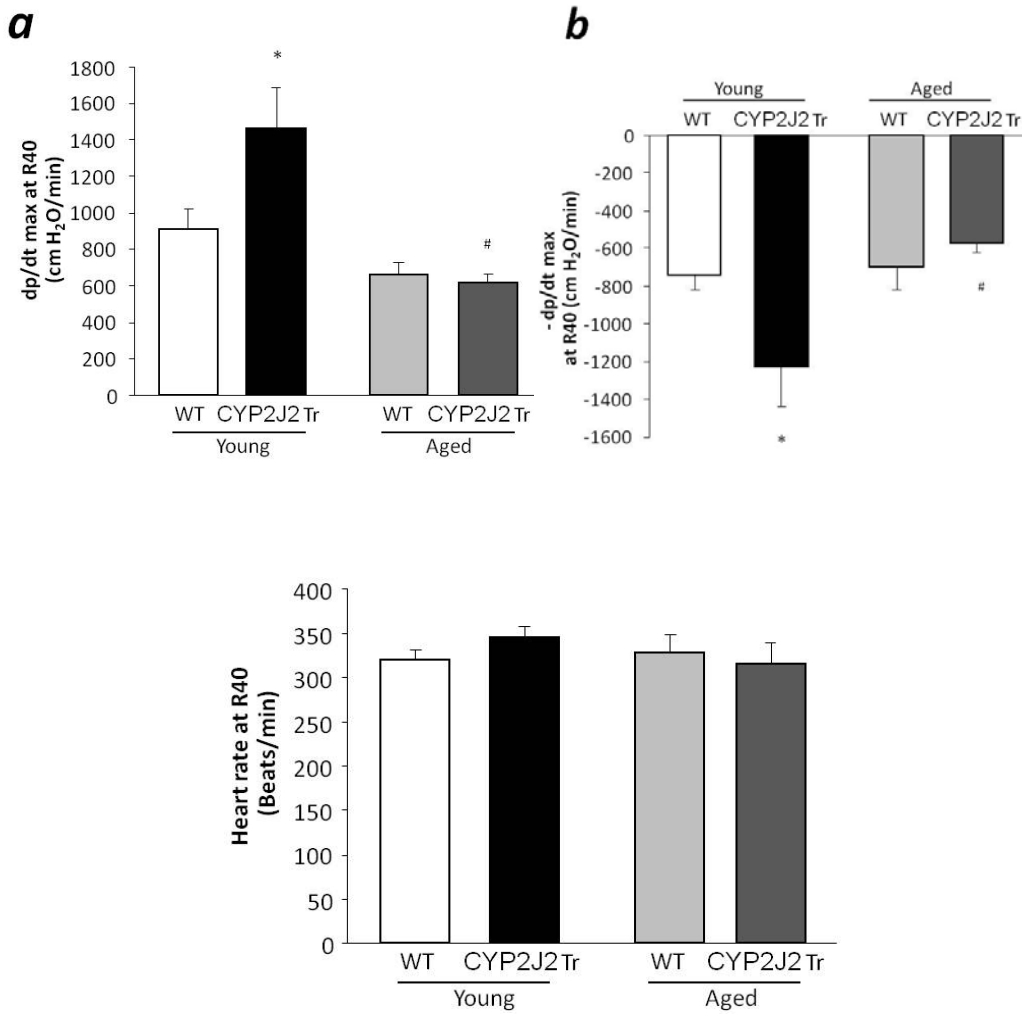

Figure 4.2 Cardiac parameters of young and aged CYP2J2 Tr mouse hearts. *a*. Rate of contraction, *b*. rate of relaxation and *c*. heart rate in young and aged, WT and CYP2J2 Tr hearts. Values represent mean ± SEM; n=8-15 per group; *, $p<0.05$ vs. WT; $^{\#}$, $p<0.05$ vs. young animal of same genotype

4.3.2 Post-ischemic cardiac function in young and aged sEH null mice

Consistent with our previous observations [10], sEH null and WT mice had normal baseline contractile function (Fig 4.3a). Furthermore, young sEH null hearts had improved postischemic LVDP recovery following 30min of global ischemia and 40min reperfusion compared to WT hearts (38.5% vs. 20.7%, respectively, $p<0.05$) (Fig 4.3b). Interestingly, opposite to the loss of cardioprotective effects in aged CYP2J2 Tr mice aged, improved postischemic left ventricular function was observed in aged sEH null mice (31.1% vs. 12.9%, respectively, $p<0.05$) (Fig 4.3a and Fig 4.3b). Both, young and aged, sEH null mouse hearts demonstrated significantly higher contraction and relaxation rate compared to age matched WT (Fig 4.4a and Fig 4.4b). No difference was observed in heart rate between any groups (Fig 4.4c). Taken together, these data suggest that targeted deletion of sEH but not CYP2J2 overexpression protects the aged mouse hearts against ischemia reperfusion injury.

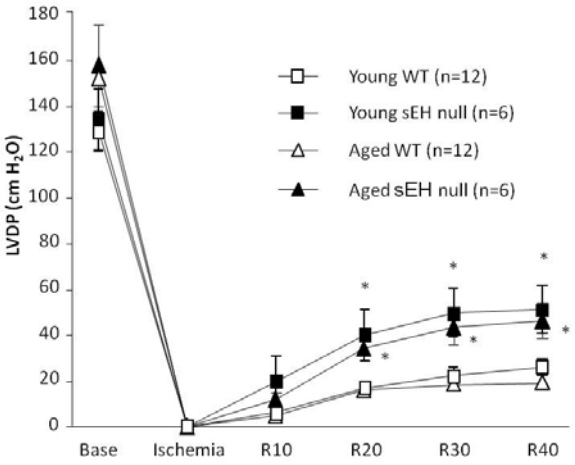

Figure 4.3a LVDP of young and aged sEH null mouse hearts. LVDP at baseline, at the end of ischemia, and at reperfusion (R10, R20, R30, and R40) in young and aged, WT and sEH null hearts. Values represent mean ± SEM; n=6-12 per group; *, $p<0.05$ vs. WT; #, $p<0.05$ vs. young animal of same genotype.

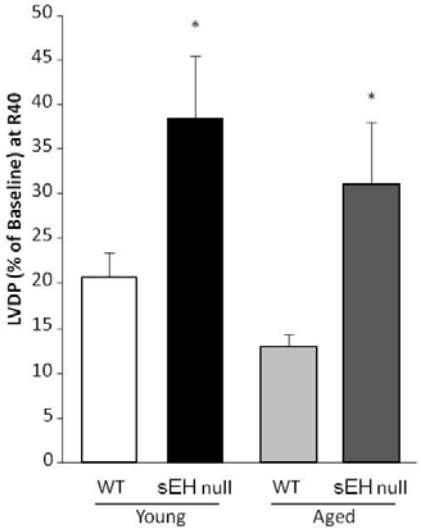

Figure 4.3b LVDP recovery of young and aged CYP2J2 Tr mouse hearts. LVDP recovery at 40min of reperfusion in young and aged, WT and sEH null hearts. Values represent mean ± SEM; n=8-15 per group; *, $p<0.05$ vs. WT.

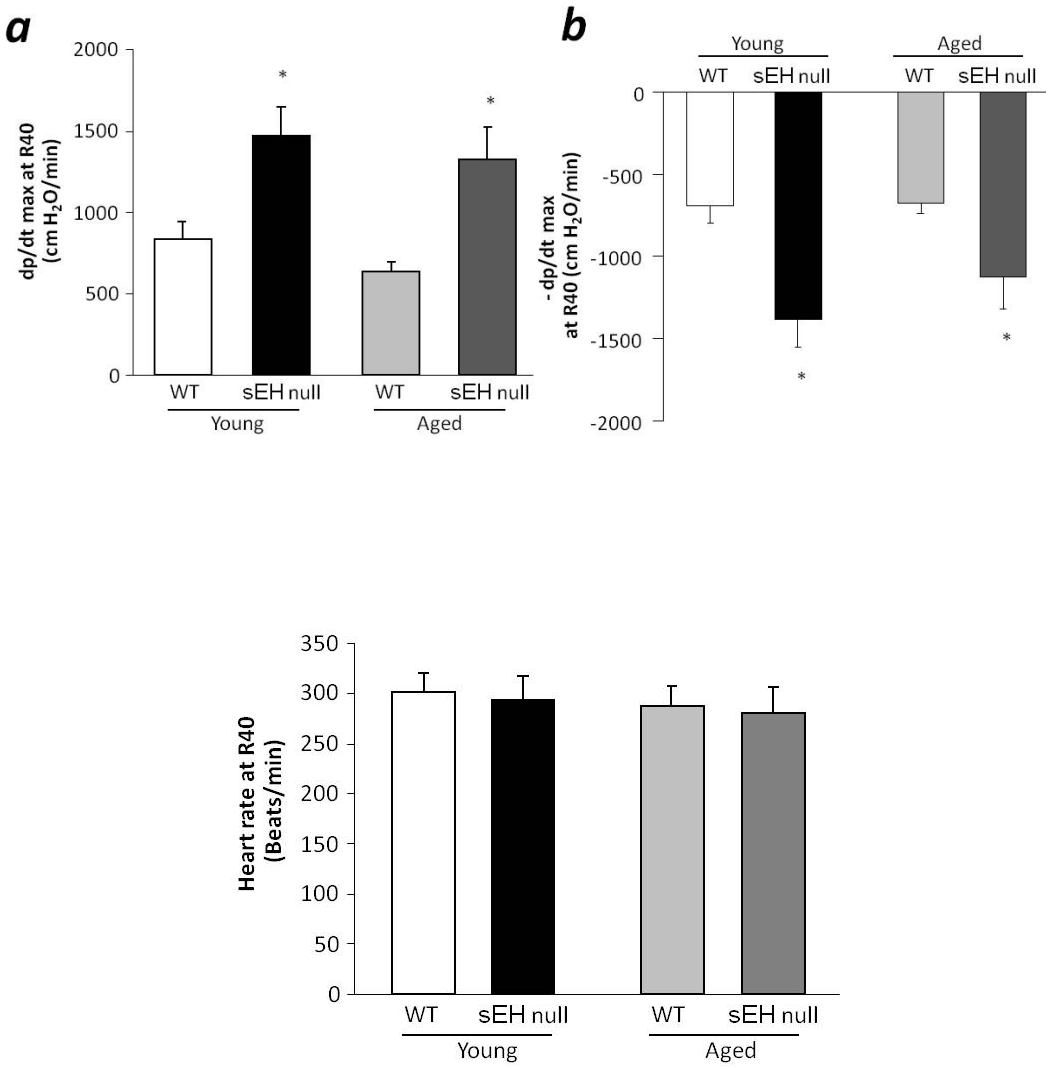

Figure 4.4 Cardiac parameters of young and aged sEH null mouse hearts. *a*. Rate of contraction, *b*. rate of relaxation and *c*. heart rate in young and aged, WT and sEH null hearts. Values represent mean ± SEM; n=6-12 per group; *, $p<0.05$ vs. WT.

4.3.3 sEHi and postischemic functional recovery in aged CYP2J2 Tr mice

Previous reports suggest that aging is associated with increased epoxide hydrolase expression in soluble fraction of the heart.[27] To study the role of sEH, we perfused the aged CYP2J2 Tr mouse hearts with soluble epoxide hydrolase inhibitor *t*-AUCB (100 nM). At this concentration *t*-AUCB has been demonstrated to have protective effects against ischemia reperfusion injury.[12] Perfusion with *t*-AUCB resulted in significant improvement of postischemic functional recovery in aged CYP2J2 Tr hearts compared to controls (33.9% vs. 15.4%, respectively, $p<0.05$) (Fig 4.5). This improvement in postischemic functional recovery was inhibited by co-perfusion of aged CYP2J2 Tr hearts with *t*-AUCB and MS-PPOH (CYP epoxygenase inhibitor) (15.9%) (Fig 4.5). These data confirms that *t*-AUCB induced cardioprotection in aged CYP2J2 Tr mice is due to inhibition of eicosanoid metabolism. Moreover, sEH inhibition and thereby EETs can protect the aged mouse hearts against ischemia reperfusion injury.

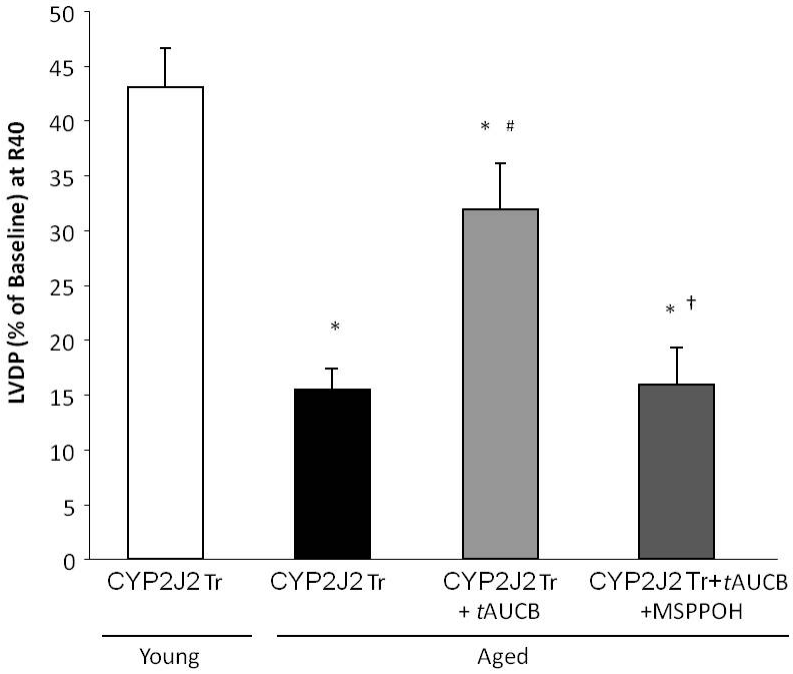

Figure 4.5 EET-induced cardioprotection in aged mice. LVDP recovery at 40min of reperfusion in aged CYP2J2 Tr mice treated with *t*-AUCB (100 nM) or *t*-AUCB (100nM) and MS-PPOH (50 μM). Values represent mean ± SEM; n=5-9 per group; *, $p<0.05$ vs. young animal of same genotype; $^{\#}$, $p<0.05$ vs vehicle control; †,$p<0.05$ vs *t*-AUCB treated.

4.3.4 sEH expression and activity analysis

To further confirm the role of sEH, we analysed cytosolic and microsomal fractions of young and aged mouse hearts for epoxide hydrolase activity and sEH expression. Interestingly, no difference was observed in sEH expression in cytosol of young or aged CYP2J2 Tr or WT hearts (Fig 4.6a). Indeed, no sEH expression was observed in young or aged sEH null mice hearts. Consistent with the protein expression, no difference was observed in the epoxide hydrolase activity in cytosol or microsomal fractions of any groups (Fig 4.6b and Fig 4.7). No epoxide hydrolase activity was observed in cytosol or microsomal fractions of young or aged sEH null heart samples (Fig 4.6b and Fig 4.7). These data confirms that the loss of protective effects in aged CYP2J2 Tr mouse hearts is not due to higher sEH activity in aged hearts. However, the improved postischemic function in aged sEH null and *t*-AUCB treated aged CYP2J2 Tr mouse hearts suggests that inhibition of EET-metabolism can protect the aged hearts against ischemia reperfusion injury.

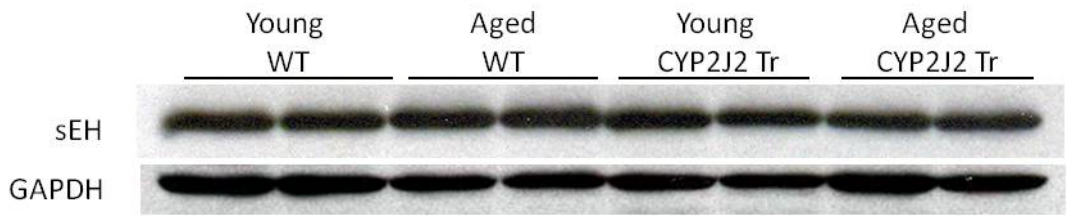

Figure 4.6a sEH expression in young and aged CYP2J2 Tr mouse hearts. Representative immunoblot of sEH in cytosol fractions of young WT, aged WT, young CYP2J2 Tr and aged CYP2J2 Tr hearts.

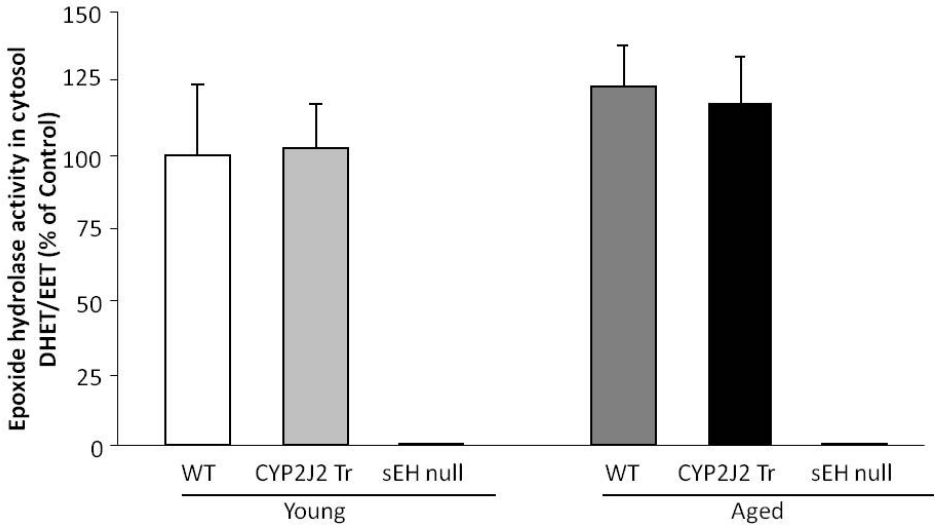

Figure 4.6b Epoxide hydrolase activity in cytosol of postischemic heart samples. Epoxide hydrolase activity in cytosol fractions of young WT, aged WT, young CYP2J2 Tr and aged CYP2J2 Tr hearts. Values represent mean±SEM; n=5 per group.

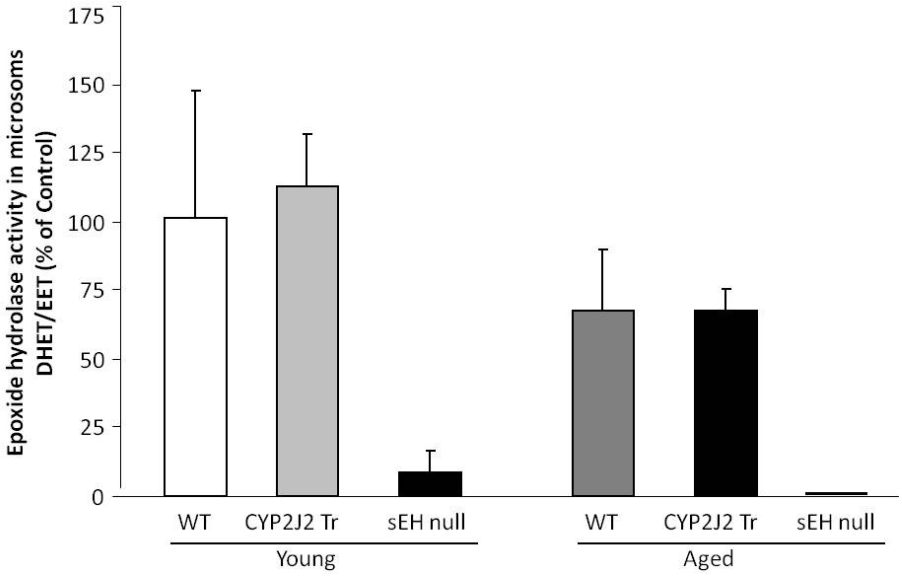

Figure 4.7a Epoxide hydrolase activity in microsomal fraction of postischemic heart samples. Epoxide hydrolase activity in microsomal fractions of young WT, aged WT, young CYP2J2 Tr and aged CYP2J2 Tr hearts. Values represent mean±SEM; n=5 per group.

4.3.5 Eicosanoid analysis

To investigate whether the synthesis of EETs was altered in aged CYP2J2 Tr mouse hearts, we analyzed CYP2J2 expression in the heart samples and measured eicosanoid levels in heart perfusates during baseline. We did not observed any difference in CYP2J2 expression in young or aged CYP2J2 Tr mouse hearts (Fig 4.8a). Moreover, levels of 14,15-EET and 11,12-EET were higher in aged CYP2J2 Tr (4.26 fold and 3.45 fold) and sEH null (4.3 fold and 3.73 fold) hearts compared to aged WT hearts (Fig 4.8b and 4.9a). Moreover, the 14,15-EET:14,15-DHET ratio was significantly higher in aged sEH null mouse hearts compared to aged CYP2J2 Tr and WT hearts suggesting lower epoxide hydrolase activity in sEH null hearts compared to CYP2J2 Tr and WT hearts (Fig 4.9b). There was no difference between aged CYP2J2 Tr and WT hearts for EET:DHET ratio (Fig 4.9b). These data suggest that aged CYP2J2 Tr and sEH null mice produce similar levels of EETs although aged sEH null mice are protected against ischemia reperfusion injury and aged CYP2J2 Tr are not.

CYP epoxygenases can metabolize linoleic acid to 9,10-epoxy-12-octadecenoate (9,10-EpOME) and 12,13-epoxy-9-octadecenoate (12,13-EpOME) which is further metabolized to dihydro-metabolites (9,10-, and 12,13-DiHOME, respectively) by sEH.[28] The detrimental effect of DiHOME on postischemic functional recovery has been reported.[17] Interestingly, aged CYP2J2 Tr mice hearts produce significantly higher

levels of 12,13-DiHOME compared to age matched WT (Fig 4.10), while aged sEH mice has significantly lower levels of 12,13-DiHOME (Fig 4.10). Thus, suggesting increased DiHOME levels in aged CYP2J2 Tr mice might be responsible for the loss of cardioprotective effects in these mice.

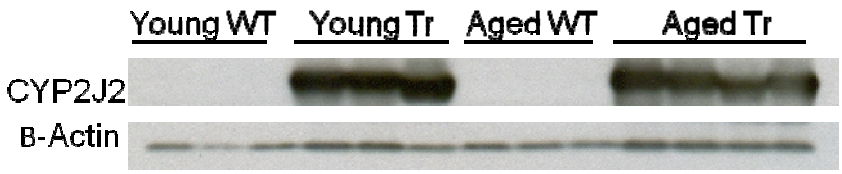

Figure 4.8a CYP2J2 expression in young and aged CYP2J2 Tr mouse hearts. Representative immunoblot of CYP2J2 in microsomal fractions of young WT, aged WT, young CYP2J2 Tr and aged CYP2J2 Tr hearts.

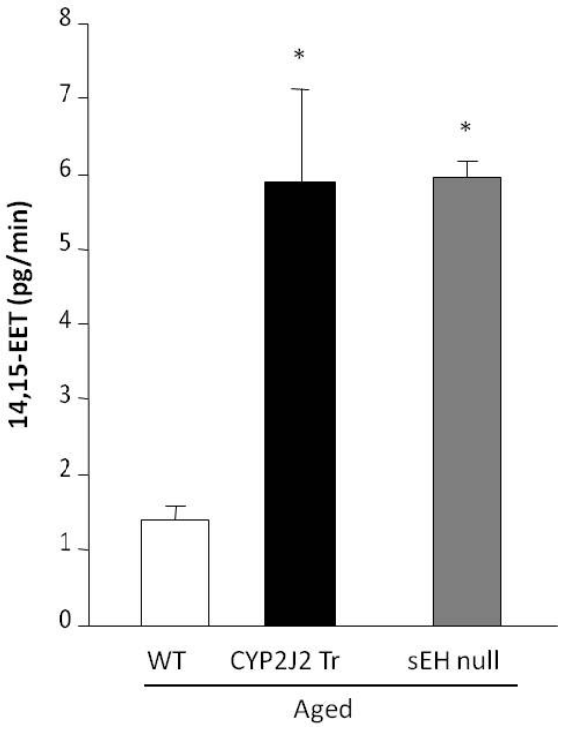

Figure 4.8b 14,15-EET levels in the heart perfusate collected during last 20 min of baseline. Values represent mean±SEM; n=3-6 per group; *, $p<0.05$ vs. WT.

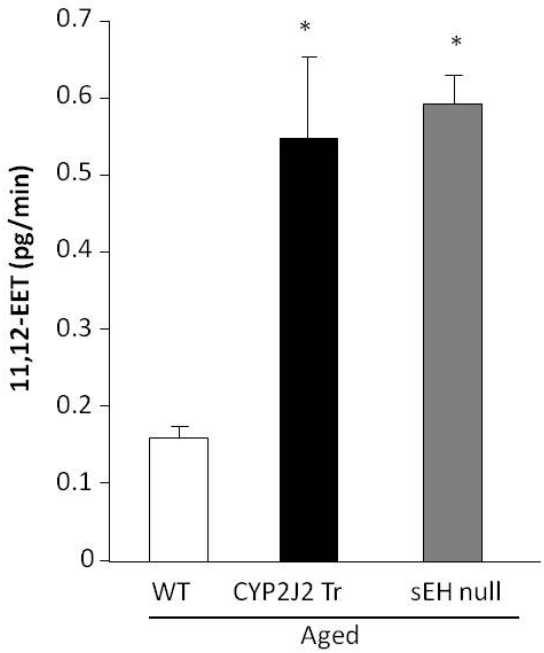

Figure 4.9a 11,12-EET levels in the heart perfusate collected during last 20 min of baseline. Values represent mean±SEM; n=3-6 per group; *, $p<0.05$ vs. WT.

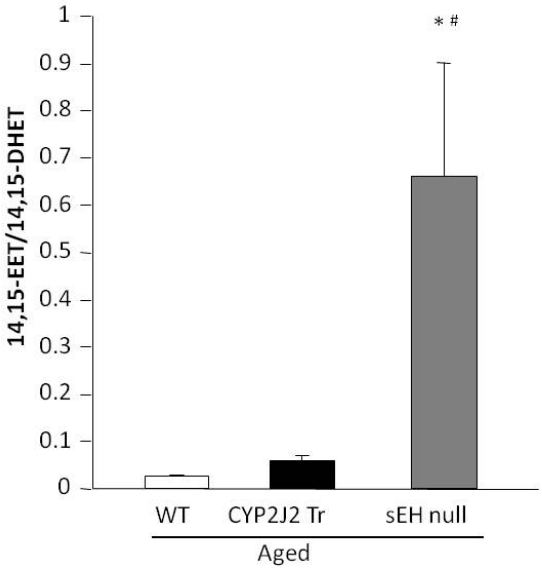

Figure 4.9b 14,15-EET:14,15-DHET ratio in the heart perfusate collected during last 20 min of baseline. Values represent mean±SEM; n=3-6 per group; *, $p<0.05$ vs. WT; #, $p<0.05$ vs. aged CYP2J2 Tr.

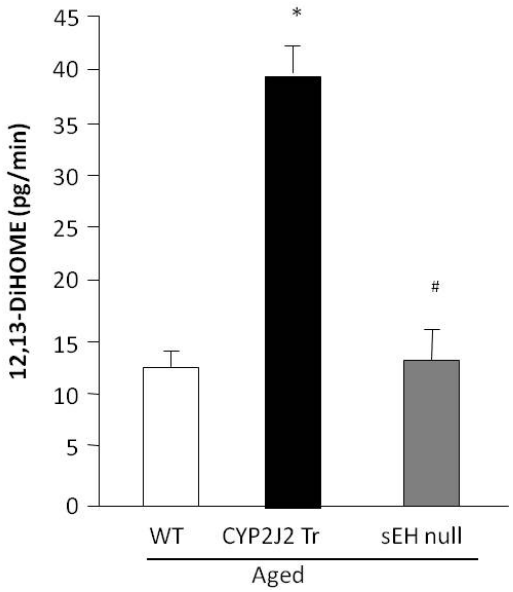

Figure 4.10 12,13-DiHOME levels in the heart perfusate collected during last 20 min of baseline. Values represent mean±SEM; n=3-6 per group; *, $p<0.05$ vs. WT; #, $p<0.05$ vs. aged CYP2J2 Tr.

4.3.6 Analysis of oxidative stress markers

Recently, Edin et al. demonstrated that endothelial expression of human cytochrome P450 epoxygenase CYP2C8 (Tie2-CYP2C8) increases susceptibility to ischemia-reperfusion injury in isolated mouse heart, potentially resulting from increased DiHOME and ROS production.[17] To investigate ROS production in aged mice, we analyzed levels of protein carbonylation in postischemic heart samples, as a marker of oxidative stress.[29] Young CYP2J2 Tr mice had significantly lower levels of protein carbonyl as compared to age matched WT (Fig 4.11a). No difference was observed in protein carbonyl levels between young and aged WT. Interestingly, aged CYP2J2 Tr mice had significantly higher protein carbonyl levels compared to age matched controls (Fig 4.11a). Furthermore, the loss of cardioprotective effects in aged CYP2J2 Tr hearts was accompanied by a significant increase in protein carbonyl levels compared to hearts from young CYP2J2 Tr mice Fig 4.11a). Further evidence for increased stress was observed in perfusate from preischemic hearts of aged mice. A significant increase in 8-iso-PGF2α levels, a marker of ROS, was observed in aged CYP2J2 Tr hearts compared to age matched WT (Fig 4.11b). Conversely, perfusate from aged sEH null hearts had lower levels of 8-iso-PGF2α compared to aged CYP2J2 Tr mice (Fig 4.11b). Thus suggesting a higher level of oxidative stress occurs in aged CYP2J2 Tr hearts.

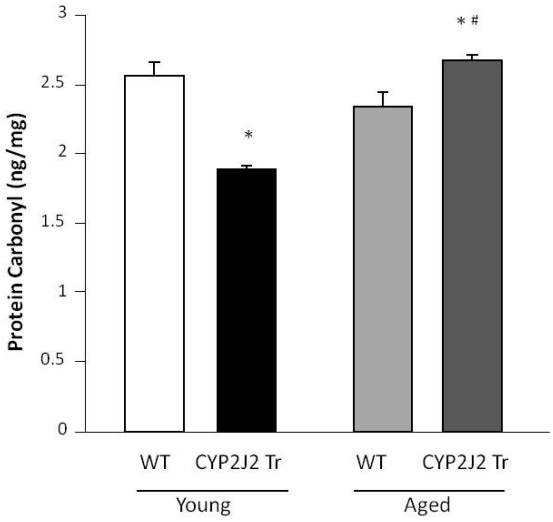

Figure 4.11a Protein carbonyl levels in postischemic heart samples. Protein carbonyl levels were measured in the cytosolic fraction of the ischemia reperfused hearts of young WT, aged WT, young CYP2J2 Tr and aged CYP2J2 Tr mice. Values represent mean ± SEM; n=3 per group; *, $p<0.05$ vs. WT; #, $p<0.05$ vs. young animal of same genotype.

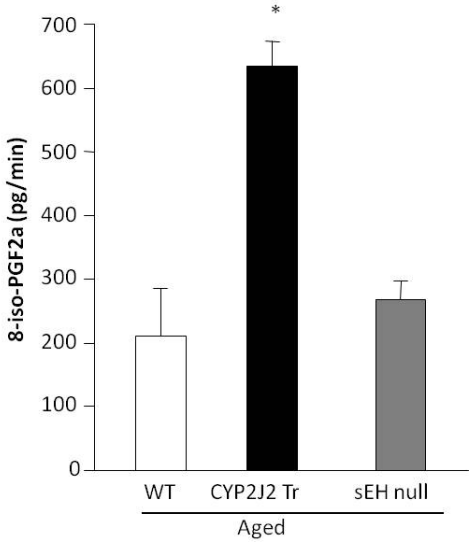

Figure 4.11b 8-iso-PGF2α levels in the heart perfusate collected during last 20 min of baseline. Values represent mean±SEM; n=3-6 per group; *, $p<0.05$ vs. WT.

4.3.7 Protein phosphatase 2A (PP2A) activity analysis

Previous studies have demonstrated that EETs increases PP2A activity in muscle cells.[30-31] Hence, we analyzed PP2A activity in young and aged CYP2J2 Tr, sEH null or WT heart samples. Significant increase in PP2A activity was observed in young CYP2J2 Tr and young sEH null mouse hearts compared to age matched WT hearts (Fig 4.12). Aged CYP2J2 Tr mouse hearts failed to demonstrate increase in PP2A activity compared to age matched WT (Fig 4.12). However, significant increases in PP2A activities were observed in hearts from aged sEH null mice and aged CYP2J2 Tr mice perfused with *t*-AUCB compared to aged matched controls. In addition, co-perfusion of *t*-AUCB with MS-PPOH in aged CYP2J2 Tr hearts attenuated the increased PP2A activity (Fig 4.12). AMPK is a target for PP2A, as such, to confirm changes in PP2A activity, we analyzed p^{Thr172}-AMPK expression in these heart samples. Consistent with increased PP2A activity in young CYP2J2 Tr mouse hearts, significant decrease in p^{Thr172}-AMPK expression was observed while higher p^{Thr172}-AMPK expression was observed in aged CYP2J2 Tr mouse hearts (Fig 4.13).

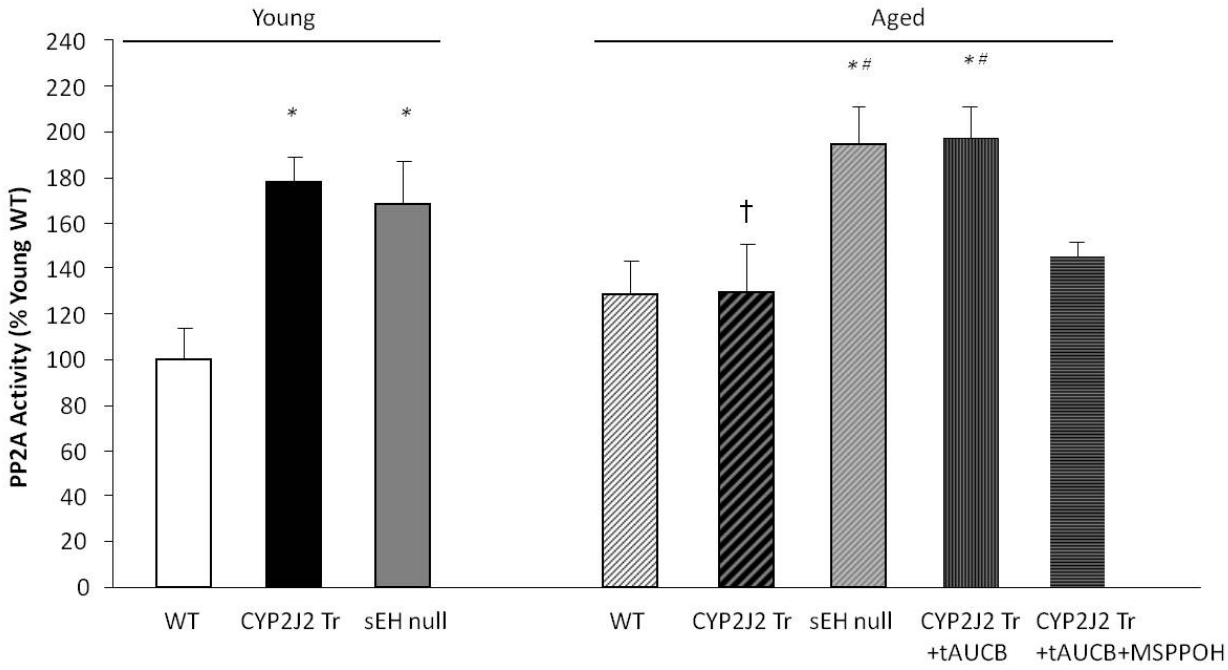

Figure 4.12 PP2A activity was measured in the cytosolic fractions of the ischemia reperfused hearts. Values represent mean ± SEM; n=3-7 per group; *, $p<0.05$ vs. age matched WT; #, $p<0.05$ vs aged CYP2J2 Tr; †,$p<0.05$ vs young animal of same genotype.

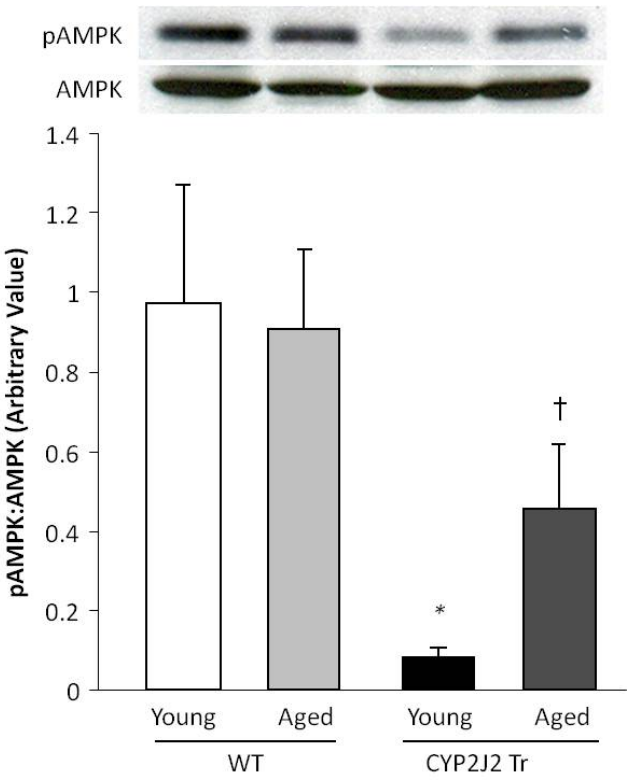

Figure 4.13 pAMPK expression in young and aged CYP2J2 Tr mouse heart samples. Representative immunoblot and densitometry demonstrating expression of pAMPK in cytosolic fraction of ischemia reperfused mice hearts. Values represent mean ± SEM; n=6 per group; *, $p<0.05$ vs. age matched WT; †,$p<0.05$ vs young animal of same genotype.

4.4 Discussion

Numerous reports have demonstrated the cardioprotective effects of EETs in young animal models; however, it remains unknown whether EETs can trigger cardioprotection in aged animals.[9-10, 14] Evidence suggests that many cardioprotective strategies, such as ischemic and anesthetic preconditioning, are lost in aged animals.[24, 32-33] In the current study, we demonstrate that EETs can provide cardioprotection towards IR injury in aged animals. The decreased postischemic functional recovery observed in aged mice with cardiomyocyte specific over-expression of CYP2J2 was attributed to increased DiHOME levels, oxidative stress and activation of PP2A. Importantly, our data demonstrated that the targeted deletion of sEH or pharmacological inhibition of sEH resulted in improved postischemic recovery in aged mice. Taken together, these data suggest inhibition of sEH can be an effective strategy for protecting aged hearts against I/R injury.

There is a growing body of evidence suggesting the effectiveness of cardioprotective strategies, including preconditioning, postconditioning and pharmacological conditioning, decreases with increasing age.[24, 32-33] Schulmann et al reported the effect of aging on various cardioprotective strategies toward I/R injury.[32] In the study, the authors utilized young , mid-aged and aged rats to assess the infarct size reduction properties of ischemic preconditioning and pharmacological conditioning mimetics such as selective adenosine A1 agonist (CCPA),

PKC analogue (DOG) and mitochondrial K_{ATP} channel opener (diazoxide). Ischemic preconditioning effectively reduced the infarct size following ischemia reperfusion in young animals; however, it was less effective in middle-aged animals and ineffective in aged animals. Similarly, pharmacological conditioning mimetics were ineffective in aged animals.[32] Other animal models have demonstrated blunted effects of preconditioning, postconditioning and pharmacological conditioning against IR injury upon aging.[24, 33] Similarly, we observed loss of cardioprotection in aged CYP2J2 Tr mice. Interestingly, aged sEH null mice and *t*-AUCB treated age CYP2J2 Tr mice demonstrated improved postischemic left ventricular function. Together, our results suggest that inhibition of sEH can be an effective strategy to induce cardioprotection in aged myocardium.

Several factors may be involved in loss of cardioprotection in aged CYP2J2 Tr mice including increased epoxide hydrolase activity, increased production of detrimental molecules and impaired response to cardioprotective stimuli. Studies demonstrated increased epoxide hydrolase expression in the soluble fraction of the left ventricle of middle aged and aged rats.[27] Therefore, we hypothesized that increased metabolism of EETs due to increased epoxide hydrolase activity is responsible for the loss of cardioprotection in aged CYP2J2 Tr mice. Improved postischemic function in aged CYP2J2 Tr mice following perfusion with *t*-AUCB further supported the hypothesis. However, no

difference in sEH expression or activity in the present study suggested the involvement of factors other than increased activity of sEH in the loss of cardioprotection.

CYP2J2 can effectively metabolize linoleic acid to EpOMEs, which have been shown to produce adverse cardiac effects in various animal models.[34-36] In anaesthetized dog model, infusion of EpOMEs causes significant reduction in aortic flow, aortic pressure, contractility and heart rate in dose dependent manner.[35] However, in absence of sEH activity, EpOME do not exert their cytotoxic effects.[37] Reports suggest that the toxic effects of EpOMEs are due to their metabolites, DiHOME, and therefore EpOMEs are considered as protoxins.[36-38] DiHOMEs but not EpOMEs inhibits electrical activity in isolated rat ventricular myocytes.[36] In addition, sEH null mice have been demonstrated to have significantly higher EpOME:DiHOME ratios and are protected against IR injury suggesting that DiHOMEs can be detrimental to the heart.[10] In experimental models of myocardial ischemia, DiHOME levels rise from 1–3 μg/g of tissue to 4–22 μg/g of tissue.[39] DiHOMEs but not EpOME depress Na^+ channels, K^+ channels and trigger mitochondrial dysfunction.[36, 40-41] DiHOMEs are responsible for a massive burst of ROS in vascular endothelial cells.[42] Furthermore, perfusion of isolated mice hearts with DiHOME results in decreased postischemic functional recovery suggesting DiHOME may contribute to the detrimental effects of myocardial ischemia.[17] In the present study, higher levels of DiHOME in

aged CYP2J2 Tr mice correlate with loss of cardioprotection, while aged sEH null mice had lower levels of DiHOME and better postischemic cardiac function. This data suggests that inhibition of sEH can prevent the formation of DiHOMEs and protect the heart from the detrimental effects of DiHOMEs. Improved postischemic functional recovery in *t*-AUCB treated aged CYP2J2 Tr mice, further suggests that DiHOMEs are involved in the loss of cardioprotection in aged CYP2J2 mice. Therefore, inhibiting sEH can increase EET-levels and potentially prevent the formation of DiHOME, which both can contribute to cardioprotection in aged mice.

Adverse effects of CYP epoxygenase activity have been demonstrated in IR heart studies suggesting an increased production of ROS results from uncoupling of CYP reactions. For example, inhibition of CYP2C by sulphaphenazole reduces infarct size in isolated rat hearts. The protective effects are due to suppression of CYP2C-induced ROS production during reperfusion and reduce tissue damage.[43] Recently, Edin et al. reported increased generation of 8-iso-PGF2α in the cardiac perfusate of mice with endothelial cell specific expression of human CYP2C8 (Tie2-CYP2C8).[17] Moreover, 8-iso-PGF2α was associated with decreased postischemic functional recovery in Tie2-CYP2C8 mice, which was improved after the treatment with the ROS scavenger (N-acetyl-cysteine; NAC) or the antioxidant enzyme (superoxide dismutase).[17] Conversely, previous studies demonstrate a low tendency of CYP2J2 for

generating ROS and inhibitory effects of EETs on ROS production.[44-45] Consistent with these reports, young CYP2J2 Tr mice had significantly lower levels of protein carbonyl following IR. However, aged CYP2J2 Tr mice had significantly higher protein carbonyl levels than age matched WT and young CYP2J2 Tr. Similarly, we observed that isolated heart perfusate from aged CYP2J2 Tr mice had increased levels in 8-iso-PGF2α suggesting higher oxidative stress in these mice. These data suggest that the aging process correlates with increased markers of oxidative stress in mice overexpressing cardiac CYP2J2.

EET-mediated signaling is known to cause activation of several protein kinases such as PI3K, Akt, PKCε and PKA, where kinase inhibition will significantly attenuate EET-mediated effects.[9-10, 14-15] In contrast, the current study demonstrated that EETs can activate PP2A, which can result in dephosphorylation and inhibition of protein kinases such as Akt, AMPK and Erk.[46] Limited evidence for EET activation of PP2A has been demonstrated in vascular smooth muscle cells.[30-31] We observed loss of PP2A activation in aged CYP2J2 Tr mice that is consistent with the loss of EET-induced cardioprotection. Moreover, PP2A activation was evident in young and aged sEH null mice and aged CYP2J2 Tr mice treated with *t*-AUCB, demonstrating correlation between increased PP2A activity and improved postischemic functional recovery. Low PP2A activity in aged CYP2J2 Tr mice co-perfused with *t*-AUCB and MS-PPOH further confirms that decreased postischemic functional recovery due to inhibition EET-

synthesis involves decreased PP2A activity. Together, our results indicate that decreased PP2A activity in addition to DiHOME and ROS may be responsible for loss of cardioprotection in aged CYP2J2 Tr mice.

The role of AMPK in I/R injury is extensively studied; however, it remains controversial whether inhibition of AMPK is beneficial or detrimental.[47-50] In recent studies, small molecule activators of AMPK during ischemia protects against myocardial ischemic injury [48] and mice with cardiomyocyte-specific over-expression of a mutant AMPKα_2 subunit (kinase dead) are susceptible to I/R injury.[49] In contrast, mouse hearts expressing dominant negative α2-subunit of AMPK are not energetically compromised and do not have impaired but may in fact have improved recovery of function after ischemia.[50] Another study has demonstrated that inhibition of AMPK restores cardioprotection of adenosine against antecedent ischemia.[47] Moreover, the study reported significant inhibition of phospho^{Thr172}AMPK expression and AMPK activity by adenosine at the end of reperfusion.[47] Similarly, in the present study, we observed decreased phospho^{Thr172}AMPK expression in young CYP2J2 Tr hearts compared to age matched WT at the end of reperfusion. This could be due to increased activation of PP2A that can effectively dephosphorylate phospho^{Thr172}AMPK. Further studies are required to investigate the role of AMPK and PP2A in EET-induced cardioprotection.

4.5 Conclusion

In conclusion, we report abrogated cardioprotective response of EETs in aged CYP2J2 Tr mice and that could be prevented by inhibition of sEH. Moreover, loss of cardioprotection in aged CYP2J2 Tr mice is due to increased DiHOME levels, oxidative stress and decreased effectiveness of EETs all of which can be prevented by inhibition of sEH. Together, EET can effectively protect the aged mouse hearts against ischemic reperfusion injury.

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Chapter 5

General Discussion and Conclusion

According to the latest report available by Heart and Stroke Foundation of Canada and Statistics Canada, cardiovascular disease accounted for 29% deaths in Canada in 2008. Around 69,500 deaths occurred due to CVD and that means every 7 minutes someone died from CVD in 2008. Among the deaths occurred due to CVD, 54% of deaths were attributed to the ischemic heart diseases. (http://www.heartandstroke.ab.ca/site/c.lqIRL1PJJtH/b.3650897/k.35F8/Statistics.htm) These statistics demonstrate the importance of research focused on understanding the pathophysiology of ischemic heart diseases as well as investigating the mechanisms of cardioprotection induced by various pharmacological agents. These efforts will facilitate the discovery of new molecules that can be used for the treatment of ischemic heart diseases and reduce the burden of these diseases.

This dissertation has investigated the cardioprotective properties of sEHi, the mechanism of cardioprotection induced by EETs and also explored the protective effects of EETs in young and aged animals. First, we investigated cardioprotective effects of a novel sEHi, *t*-AUCB, using isolated perfused mouse heart model. We demonstrated that *t*-AUCB was cardioprotective when used in nanomolar concentration and these effects were mediated through EETs. Furthermore, we confirmed the involvement of PI3K-Akt pathway in EET-induced cardioprotection. Therefore, inhibitors of sEH can be used as pharmacological agents to achieve EET-

induced cardioprotection and investigate the mechanisms underlying the cardioprotective effects.

The second major study investigated mechanisms underlying EET-induced cardioprotection. Our preliminary results demonstrated that targeted disruption of the *Ephx2* gene resulted in increased BNP mRNA expression and 14,15-EEZE inhibited this effect. Based on these observations we hypothesized that BNP may play a role in EET-induced cardioprotection. In that study, we reported that targeted deletion of Ephx2 gene or treatment with exogenous 11,12-EET causes increase in BNP mRNA and protein expression following ischemia-reperfusion resulting in improved functional recovery. Moreover, we demonstrated a critical role of BNP and GSK3β in mediating the cardioprotective effect of EETs. These data suggested that EET-mediated cardioprotection involves two pathways, PI3K/Akt and BNP-PKCε-mediated signaling, which converge on the mitochondria.

Cardioprotective effects of EETs have been reported in young animal models; however, there is a lack of information about EET-induced cardioprotection in aged animals. The third major study in this dissertation investigated the effect of aging on EET-induced cardioprotection. We reported that targeted deletion of *EPHX2* gene and pharmacological inhibition of sEH is protective against I/R injury in young as well as aged mice. On the contrary, cardiomyocyte specific over-expression of CYP2J2 does not protect the aged heart against I/R injury. The loss of

cardioprotection in aged CYP2J2 Tr mice is attributed to increased DiHOME levels, ROS and decreased activation of PP2A. Furthermore, inhibition of sEH prevents the increase in DiHOME and ROS and also maintains the PP2A activity in aged mice. Together, our data suggest that increasing EETs through inhibition of sEH can be an effective strategy for protecting aged mice heart against I/R injury. This chapter will discuss the results in this dissertation in context of the current literature.

5.1 sEH inhibition and Cardioprotection

As described in the introduction (Chapter 1), current pharmacological treatment of ischemic heart disease focuses mainly on improving hemodynamic parameters and reducing myocardial O_2 demand, as such there remains a need for new pharmacological agents that directly protect the heart against I/R injury. EETs are potential endogenous molecules that protect the heart against I/R injury. Majority of the studies investigating the effects of EETs on I/R injury have reported cardioprotection in response to increased levels of EETs.[1-6] Various strategies have been used to elevate EET-levels in the heart and study the protective effects against IR injury. These include: 1) treatment of the WT animals with exogenous EETs, [7-8] 2) increase EET-synthesis in the heart by cardimyocyte specific over-expression of CYP2J2, [3] 3) prevention of EET-metabolism by targeted deletion of *Ephx2* gene, [2, 4] and 4) pharmacological inhibition of sEH to prevent metabolism of EETs.[2]

Based on the data generated from this thesis and current literature, development of stable EET-analogues and pharmacological inhibitors of sEH are very good strategies to protect the heart against ischemic heart diseases. Various generations of sEHi have been developed and tested for cardioprotective effects against I/R injury. The first generation of sEHi was the epoxide-containing compounds that were the substrates of epoxide hydrolase. They had a transient inhibitory effect *in vitro*; however, they were not effective *in vivo*.[9] The next generation of sEHi (1,3-disubstituted urea derivatives) were stable and more potent sEHi than the first-generation compounds.[9-10] Few studies have reported cardioprotective effects of the second-generation sEHi (AUDA and AUDA-BE).[2] Nonetheless, these sEHi compounds have several drawbacks, which include limited oral bioavailability, instability due to rapid metabolism and poor physical properties such as low water solubility.[11] As such AUDA or AUDA-BE cannot be used orally but with very careful formulation of AUDA and its derivatives can be used orally for in vivo studies in animals.[2, 12] The new generation of sEHi (*t*-AUCB, c-AUCB, TUPS etc.) has improved water solubility and stability than the previous generation of sEHi.[11] Moreover, the new generation inhibitors are more potent than AUDA and its derivatives.[11] The data in this dissertation (chapter 2) demonstrate protective effect of a stable, water soluble and potent inhibitor of sEH, *t*-AUCB. Moreover, the data indicate that inhibition of sEH by *t*-AUCB lead to a dramatic improvement of the post-ischemic

cardiac function that is comparable to genetic knockout of sEH. The fact that both the chemical and genetic knockout experiments resulted in similar improvements in function indicates that a near maximum effect for a sEHi was reached with *t*-AUCB in the animal model used. However, neither approach resulted in full return to normal function indicating that additional approaches could be helpful in restoring cardiac function.

Consistent with our results, another study has reported cardioprotective effect of *t*-AUCB using *in vivo* infarction in mouse model of I/R injury.[13] In the study, animals were treated with *t*-AUCB in drinking water for 3 days before inducing 45 min ischemia and followed for 3 weeks to study the recovery. Ischemia caused marked decrease in EETs/DHET ratio that was improved by treatment with *t*-AUCB. Consistent with increased EETs/DHET ratio, *t*-AUCB treated animals demonstrated significant decrease in infarct size, improvement in post-ischemic cardiac function, reduction in cardiac remodeling, reduction in cellular apoptosis and also prevented the cardiac arrhythmias following myocardial ischemia.[13] These results are consistent with our isolated perfused heart model experiments (chapter 2) where we observed improved post-ischemic cardiac function and reduced infarct size in heart perfused with *t*-AUCB.

Recently, our group has reported a novel agent (UA-8) with dual properties of sEH inhibitory properties and EET-mimetic properties.[14] The cardioprotective effects of UA-8 were mainly attributed to structural

similarity to EETs.[14-15] Particularly, UA-8 has a negatively charged carboxylic acid group at carbon-1 and a cis-$\Delta^{8,9}$- olefin for EET-mimetic activity.[14-15] The postischemic functional recovery observed with UA-8 was comparable to the recovery observed in *t*-AUCB perfused hearts in Chapter 2. Nevertheless, increased resistance against autooxidation, esterification and degradation provides a significant advantage for UA-8 compared EETs and other sEHi.[15] Together, these results support our proposal of development of a novel pharmacological sEHi as a cardioprotective molecule to directly prevent the heart against I/R injury.

5.2 Role of BNP in EET-induced cardioprotection

Numerous studies, including the study presented in chapter 2, have demonstrated cardioprotective effects of EETs and also reported mechanisms underlying EET-induced cardioprotection. Pathways of EET-induced cardioprotection involves activation of signaling molecules such as PI3K-Akt and p42/44 MAPK inhibition of proapoptotic proteins like BAD, BAX, and GSK-3β and regulation of ion channels such as K^+ channels and BK_{Ca} channels.[3-4, 16-17] Data presented in chapter 3 suggested the involvement of another molecule, BNP, in the EET-induced cardioprotective signaling pathway. Following I/R, significant increase in Nppb mRNA and BNP protein expression was observed in sEH null hearts and EET-perfused WT hearts compared to vehicle perfused WT hearts. The increase was inhibited by 14,15-EEZE confirming that EETs had a role in regulating Nppb mRNA and BNP protein expression. Similarly, Xiao

et al recently demonstrated that over-expression of CYP2J2 by adeno-virus transfection increases ANP mRNA and protein expression in the heart of spontaneously hypertensive rats.[18] The increased mRNA and protein expression correlates with increased plasma and urinary EET-levels. The authors concluded that overexpression of CYP2J2 attenuate the development of hypertension and improve heart function in spontaneously hypertensive rats and the effects are mediated through ANP.[18] While the mechanism for EET-mediated activation of BNP is not known, other report [18] and the data presented in chapter 3 indicate that EETs may be the endogenous regulator of natriuretic peptide system.

The mechanisms by which EETs up-regulate BNP mRNA and protein expressions are not known. However, there are two possibilities for increase in BNP mRNA expression, either induction of mRNA synthesis or inhibition of degradation of mRNA. BNP gene expression can be induced in response to various factors such as stress, ischemic injury, phenylephrine, thyroid hormones, endothelin-1, β-adrenergic stimulation and proinflammatory cytokines, such as interleukin-1β.[19] Interestingly, up-regulation of BNP gene in response to β-adrenergic stimulation is mediated through signaling that involves activation of cAMP, PI3K-Akt, MAPK and CaMKII.[19] Similar signaling molecules are activated by EETs; however, data presented in chapter 3 suggests that PI3K is not involved in EET-mediated induction of BNP gene. Similar to the β-adrenergic stimulation, EETs are known to increase cAMP levels by

stimulating putative EET-receptor [20-21] and cAMP regulates the gene transcription by trans-acting factors that bind to the cAMP-response element (CRE) of target genes.[19, 22] Therefore, it is possible that EETs may up-regulate BNP mRNA through cAMP. Alternatively, stabilization of Nppb mRNA via an MAPK and PKC dependent mechanism results in increased levels as demonstrated in neonatal rat cardiomyocytes.[23] Further studies are required to investigate the mechanisms by which EETs regulate BNP gene expression.

Studies suggest that BNP can protect the heart against I/R injury.[24-25] The effects of BNP in the heart are mediated through a natriuretic peptide receptor (NPR-A) and these effects can be blocked by inhibition of NPR-A. In chapter 3, we demonstrated that NPR-A antagonist (A71915) inhibits improved post-ischemic functional recovery in sEH null hearts and 11,12-EET treated WT hearts confirming the role of BNP as a mediator of EET-induced cardioprotection. Furthermore, we investigated signaling involved in BNP and EET-induced cardioprotection. The intracellular domain of the NPR-A receptor is a guanylyl cyclase domain.[26] Activation of this receptor by BNP results in increased intracellular cGMP, a second messenger.[25-26] Increased intracellular cGMP causes activation of a serine threonine kinase, protein kinase G (PKG) that prevents the opening of mPTP and protect the heart against I/R injury. [26-30] Moreover, this inhibitory effect of cGMP and PKG on mPTP can be blocked by specific mitoK_{ATP} channel inhibitor (5-HD) and

PKCε inhibitor (εV_{1-2}) suggesting the role of mitoK_{ATP} and PKCε in BNP mediated cardioprotection.[27-28] Together, BNP induced cardioprotective signaling involves activation of NPR-A, cGMP, PKG, PKCε, mitoK_{ATP} and inhibition of mPTP. Similarly, EET-induced cardioprotection is mediated through activation of mitoK_{ATP} channels and inhibition of mPTP opening.[4, 31] However, the mechanism of EETs mediated activation of mitoK_{ATP} is not known. Studies suggest that activation of PKCε by various cardioprotective stimuli results in phosphorylation and translocation of PKC to the mitochondria.[32] On the mitochondrial membrane, PKC interact with mitoK_{ATP} and proteins of mPTP and prevent the opening of mPTP.[27, 33] Our data from chapter 3 suggests that BNP and EETs increase phosphorylation of PKCε in cytosol and mitochondrial fractions. Overall, the data presented in chapter 3 indicates that BNP is involved in EET-induced cardioprotective signaling and the effects are mediated through PKCε.

5.3 Role of PI3K in EET and BNP-induced cardioprotection

EET-induced activation of PI3K has been reported in various cell and animal models. Previous studies demonstrated that PI3K inhibitors block EET-induced cardioprotective response.[4, 6, 17] Consistent with the previous reports, in this dissertation, we demonstrated that PI3K inhibitors inhibit EET-induced cardioprotecion in sEH null or WT hearts perfused with 11,12-EET or *t*-AUCB (Chapter 2 and 3). Moreover, in

Chapter 3, we reported involvement of at-least two different pathways in EET-induced cardioprotection 1) PI3K-Akt pathway and 2) BNP-NPR-A pathway. Involvement of multiple pathways in EET-induced cardioprotection is not new. Bodiga et al reported that endogenous or exogenous EETs protect the myocardium and the mechanism of cardioprotection involves at-least two pathways 1) PI3K and 2) K_{ATP} channels.[17] As discussed earlier, K_{ATP} channels have been demonstrated to play important role in BNP-induced cardioprotection and treatment with K_{ATP} channel blocker glibenclamide or 5-hydroxy dodicanoic acid results in loss of cardioprotection induced by BNP.[25] Therefore, the two pathways involved in EET-induced cardioprotection could be 1) PI3K-Akt and 2) BNP-NPRA-K_{ATP} channels.

5.4 EET-induced cardioprotection in aged animals

The study presented in Chapter 4 examined the effect of aging on EET-induced cardioprotection in two different models with increased EET-levels in the heart, sEH null and CYP2J2 Tr mice. In chapter 4, we reported loss of EET-induced cardioprotective response in aged CYP2J2 Tr mice but not in aged sEH null mice. Moreover, we demonstrated that inhibition of sEH could prevent the loss of cardioprotection in aged CYP2J2 Tr mice. This data suggest that the loss of protective effect in aged CYP2J2 Tr mice could be due to 1) increased metabolism of EETs due higher activity of sEH, 2) decreased synthesis of EETs or 3) decreased effectiveness of EET-induced signaling in aged CYP2J2 Tr

mice. Previous report on proteomics analysis of young and aged mice hearts suggested that epoxide hydrolase expression increases in middle aged and aged mice [34] and this can explain the loss of protective effect in aged CYP2J2 Tr mice but not in aged sEH null mice. This can also explain why sEHi prevented the loss of cardioprotection in aged CYP2J2 Tr mice. However, this possibility was turned down by the EH activity analysis that demonstrated no difference in EH activity between young and aged animals. Opposite to the earlier report, we observed no difference in sEH expression between young and aged CYP2J2 Tr and WT mice, suggesting increased sEH activity is not involved in loss of cardioprotection in aged CYP2J2 Tr mice. Further, we measured eicosanoid levels in the heart perfusate of aged mice to investigate the effect of aging on eicosanoid synthesis in these hearts. Significantly higher EET-levels in aged CYP2J2 Tr and aged sEH null mice compared to aged WT indicated that lack of EET-synthesis is not involved in the loss of cardioprotection. Surprisingly, aged CYP2J2 Tr mice have higher DiHOME and 8-iso-PGF2α compared to age matched WT or sEH null mice. DiHOME has been demonstrated to cause impairment of heart function.[35] Moreover, increased 8-iso-PGF2α indicates higher oxidative stress and oxidative stress has also been demonstrated to be associated reduced postischemic functional recovery in mice.[35-36] Protein carbonyl levels in the young and aged hearts confirmed higher oxidative stress in the aged CYP2J2 Tr mice. Therefore, accumulation of DiHOME and/or

oxidative stress might be responsible for the loss of cardioprotection in aged CYP2J2 Tr mice. Moreover, accumulation of DiHOME can be prevented by inhibition of sEH [37] and therefore perfusion of aged CYP2J2 Tr mice with *t*-AUCB results in improved post-ischemic functional recovery.

An impaired response to a cardioprotective stimulus in aged hearts may also originate from decreased ligand availability or blunted responses in terms of activation or inhibition of a signaling molecule.[38-39] For example, studies analyzing the cardioprptective effects of bradykinin receptor suggests that the blunted cardioprotective response of bradykinin in aged rat hearts is due to decreased expression of the bradykinin receptors 2 in aged (24 months) rat hearts.[39] On the other side, loss of adenosine-induced cardioprotection in aged hearts was not associated with a decreased expression of adenosine receptors, but rather with impaired downstream signaling elements.[40] Similarly, decreased availability of the ligand (EETs) or impaired signaling element may be accountable for the loss of cardioprotective effects in aged CYP2J2 Tr mice. While decreased availability of EETs is not responsible for the loss of cardioprotection in aged CYP2J2 Tr mice, impaired signaling might be involved. Considering the current knowledge about EET-cardioprotective signaling, EET-induced cardioprotection may involve activation of a G-protein coupled receptor (GPCR) and the effects are mediated through $G_{\alpha s}$ subunit.[21, 41] Reports suggest decreased effectiveness of many

GPCR- $G_{\alpha s}$ associated pathways upon aging [42-43] and if EETs activate a GPCR, there is a possibility that a similar decrease in effectiveness of the EET-cardioprotective signaling may cause loss of cardioprotection in aged CYP2J2 Tr mice. Other studies and our results in chapter 4 demonstrated that EETs can activate PP2A. Higher PP2A activity in CYP2J2 Tr and sEH null mice suggested higher EET-effectiveness in these animals. Aged CYP2J2 Tr mice had decreased effectiveness of EETs which was improved upon treatment with *t*-AUCB. Moreover, we did not observe loss of EET-induced PP2A activation and cardioprotection in aged sEH null mice. Inhibition of sEH, either genetic or pharmacological, elevates EET-levels and prolongs the presence of EETs. This prolonged higher level of EETs may be required to activate cardioprtoective signaling and achieve cardioprotection in aged animals. This data further support our hypothesis that aging causes decrease in sensitivity of EET-induced signaling and in addition to DiHOME and oxidative stress decreased PP2A activation is responsible for loss of cardioprotection in aged CYP2J2 Tr mice.

5.5 Conclusion

In conclusion, the studies described in this dissertation demonstrate that modulating EET-levels in the heart can significantly influence post-ischemic recovery of LVDP. EETs protect the heart against I/R injury and the protective effects are mediated through BNP and PI3K pathway. In addition, this dissertation demonstrates the effectiveness of EETs and sEHi in protecting the heart against I/R injury in aged animals. Specifically, the studies described in this dissertation demonstrate that:

1. Treatment with a sEHi, *t*-AUCB, improves postischemic contractile function and reduces infarct size in mice. Moreover, the cardioprotective effects of sEHi were attributed to EETs and the PI3K survival pathway. The increased potency and water solubility of *t*-AUCB over other sEHi suggest that this compound may serve as a potential therapeutic agent in myocardial I/R injury.
2. Enhanced postischemic functional recovery attributed to EETs is mediated through two different signaling pathways - PI3K/Akt and BNP/NPR-A signaling. EETs can trigger the release of BNP to act in an autocrine manner or activate the PI3K pathway to induce cardioprotection. Together the data in chapter 4 provide further evidence for the beneficial effects of EETs and suggest a novel mechanism for BNP in cardioprotection.
3. Aging causes loss of cardioprotective response of EETs in aged CYP2J2 Tr mice and that could be prevented by inhibition of sEH.

Moreover, loss of cardioprotection in aged CYP2J2 Tr mice is due to increased DiHOME levels, oxidative stress and decreased PP2A activation all of which can be prevented by inhibition of sEH. Together, EET can effectively protect the aged mouse hearts against I/R injury and sEH is a better target for protecting the hearts against I/R. Therefore, sEHi can be a potential candidate for the treatment ischemic heart diseases.

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Chapter 6

Future directions

6.1 *t*-AUCB and chronic heart failure following myocardial infarction

In this thesis, we investigated the cardioprotective effects of sEHi (*t*-AUCB) using an *ex-vivo* model of I/R injury. Furthermore, Li et al has demonstrated protective effects of *t*-AUCB using an *in-vivo* model of myocardial ischemia.[1] In that study, *t*-AUCB was used as a preventive treatment; however, there is a lack of information about the protective effects of *t*-AUCB when administered after infarct healing which is more clinically relevant model to study the cardioprotective effects. Future studies are required to investigate the *in-vivo* effectiveness of *t*-AUCB in protecting the heart against chronic heart failure following myocardial infarction.

6.2 Mechanisms underlying EET-induced BNP expression

We reported that EETs increase BNP mRNA and protein expression following I/R but the mechanisms by which EETs up-regulate BNP mRNA and protein expressions are not known. BNP mRNA expression is stimulated by various stimuli and the up-regulation of BNP gene is mediated through activation of cAMP, PI3K-Akt, MAPK and CaMKII.[2] Similar signaling molecules are activated by EETs that can play a role in up-regulation of BNP mRNA expression. For example, EETs are known to increase cAMP levels by stimulating putative EET-

receptor.[3-4] cAMP regulates the gene transcription by trans-acting factors that bind to the cAMP-response element (CRE) of target genes.[2, 5] Therefore, it is possible that EETs may up-regulate BNP mRNA through cAMP. While the data presented in chapter 3 suggests that PI3K is not involved in EET-mediated induction of BNP gene, further studies are required to investigate role of other signaling molecules in up-regulation of BNP gene.

The second possibility for increased BNP mRNA expression is by stabilization of BNP mRNA via MAPK and PKC. BNP mRNA possesses destabilizing elements (AT-rich region) which is responsible for instability of the mRNA.[6] After transcription, mRNA having AT-rich region are subjected to quick degradation.[6-7] In presence of transcription inhibitor the half life of BNP mRNA is 1hr which can be increased up to 5hr after phenylephrine treatment.[6] Phenylephrine increases stability of BNP mRNA and the effects are mediated through MAPK and PKC.[6] Further studies are required to investigate whether induction or stabilization of BNP mRNA is responsible for EET-mediated up-regulation of BNP mRNA expression.

6.3 Effects of EETs and BNP on mitochondrial in aged mice

In chapter 3 of this thesis, we demonstrated that EETs and BNP increase mitochondrial translocation of phospho^{Ser729}PKCε and decrease mitochondrial expression of GSK-3β following I/R. Translocation of

phospho^{Ser729}PKCε causes activation of mitoK_{ATP} channels, preserve mitochondrial function and protect the heart against I/R injury.[8-9] Also, decreased activity of GSK-3β prevents the loss of $\Delta\Psi m$ and protects the heart against I/R injury.[10-11] In addition, studies from other groups and our lab have demonstrated that EET-induced cardioprotection involves activation of mitoK_{ATP} channels and preservation of the mitochondrial function.[12-14] Similarly, other studies and data presented in this thesis have demonstrated that BNP induced cardioprotection also involves activation of PKCε and mitoK_{ATP} channels.[15-16] Together, this data indicates that signaling activated by EETs and BNP converge onto mitochondria, preserve mitochondrial function and induce cardioprotection. Interestingly, impaired mitochondrial function over time has been suggested as an underlying cause of cardiomyocyte loss during aging.[17] Furthermore, reports suggest that bio-energetically efficient mitochondria from young individuals become less numerous, swollen, and chronically depolarized.[17] Therefore, it is of great interest to study the effect of aging on EETs and BNP induced preservation of mitochondrial function. Moreover, role of PKCε and GSK3β in EETs and BNP induced cardioprotection in aged animal need to be investigated.

6.4 Role of AMPK in EET-induced cardioprotection

Role of AMPK in protecting the heart against I/R injury remains controversial. In recent studies reported that activation of AMPK protects the heart against I/R injury and mice with decreased AMPK activity in the

heart are susceptible to I/R injury.[18-19] On the other side, studies also report that decreased activity of AMPK in the heart is not associated with loss of functional recovery and inhibition of AMPK restores cardioprotection of adenosine against antecedent ischemia.[20-21] While the effect of AMPK in I/R injury remain controversial, there is no information about role of AMPK in EET-induced cardioprotection. In chapter 4 of this thesis, we observed decreased phospho^{Thr172}AMPK expression in response to EET-induced cardioprotection. The decreased phospho^{Thr172}AMPK could result from EET-induced increased protein phosphatase 2a (PP2A) activity. However, there is another possibility that preservation of mitochondrial function and better postischemic myocardial energetic may result in decreased phospho^{Thr172}AMPK expression in the heart protected against I/R. Further studies investigating the role of AMPK in EET-induced cardioprotection are required. To study the role of AMPK, EET-induced cardioprotection can be investigated using mice over-expressing cardiomyocyte specific dominant negative mutant of AMPK.

6.5 PP2A and EET-induced cardioprotection

PP2A inhibition has been shown to be cardioprotective whereas exogenous PP2A increases susceptibility to ischemia reperfusion injury.[22-23] Conversely, evidence suggests that PP2A inhibitor, when administered before ischemia or during ischemic preconditioning, causes loss of cardioprotective effects of ischemic preconditioning.[24] Suggesting activation of PP2A during ischemia is important for mediating

cardioprotective effects of ischemic preconditioning. PP2A mediated activation of large conductance K_{Ca} channels in response to natriuretic peptide and somatostatin has been reported in pituitary tumor cells.[25] Furthermore, EET-induced PP2A activation and role of PP2A in EET-induced large conductance K_{Ca} channel activation has been reported in vascular smooth muscle cells.[26-27] Previous studies have also demonstrated that activation of large conductance K_{Ca} channels is involved in EET-induced cardioprotection in CYP2J2 Tr mice, sEH KO mice and WT mice treated with 11,12-EET.[12-13] Together, EET-induced cardioprotection may involve activation of PP2A and possibly through activation of large conductance K_{Ca} channel. In this thesis, we observed higher PP2A activity in correlation with EET-induced cardioprotection. Studies investigating the effect of PP2A inhibitors on EET-induced cardioprotection are required to explore the role of PP2A in EET-induced cardioprotection.

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Appendix 1

Effect of ischemia reperfusion injury and epoxyeicosatrienoic acid on caveolin expression in mouse myocardium

[1]A version of this chapter has been submitted for publication: Chaudhary KR, Cho WJ, Daniel EE, Seubert JM. Effect of ischemia reperfusion injury and epoxyeicosatrienoic acid on caveolin expression in mouse myocardium. *J Mol Cell Cardiol. (2011)*.

A1.1 Introduction

Caveolae are small (50-100 nm in diameter), flask-shaped invaginations in plasma membrane that are abundant in all types of cells in cardiovascular system, including endothelial cells, smooth muscle cells, macrophages, cardiac myocytes and fibroblaststhat.[1-3] Caveolins (Cav-1, -2, and -3), structural proteins found in caveolae, serve as scaffolds and regulators of signaling proteins.[1-3] Cav-1 has been detected in rat and mouse cardiomyocytes, and demonstrated to be expressed in several cell organelles including mitochondria.[4-6] Moreover, caveolae rich fractions of plasma membrane protect mitochondria against calcium overload.[7] However, to date, role of cav-1 in regulation of mitochondrial structure and function has not been studied. On the other hand, a growing body of evidence suggests that caveolins and in particular Cav-1 is essential to maintain cardiovascular homeostasis and to mediate cardioprotective signaling.[2, 5, 8-10] Targeted deletion of Cav-1 in cardiac myocytes abolishes cardiac protection produced by preconditioning.[10] Cav-1 have been shown to be associated with Cav-3, β1- and β2-adrenergic receptors, adenylylcylase5/6 and the actin binding protein filamin in cardiac myocytes.[11] Cav-1 also interacts and regulates signaling molecules involved in cardiac protection, including GPCRs, the protein tyrosine kinase Src, phosphoinositol-3 Kinase (PI3K), Akt and protein kinase C (PKC).[2, 8] Using several independent approaches, it has been shown that these interactions are mediated through a 20-amino acid

membrane proximal region of the cytosolic amino-terminal domain termed as caveolin scaffolding domain (CSD).[11]

Arachidonic acid is an essential polyunsaturated fatty acid found in the phospholipids of cell membranes which can be released into cytosol in response to stressors such as ischemia.[12] Cyclooxygenases, lipoxygenases, and cytochrome P450 (CYP) monooxygenases metabolize free arachidonic acid to biologically active metabolites collectively known as eicosanoids.[12] Epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) are products of CYP monooxygenases that act as important cellular lipid mediators in the cardiovascular, renal and nervous systems and have been shown to have protective effects against ischemia-reperfusion injury.[12-15] EET levels can be altered by reincorporation into phospholipid membranes or metabolism to smaller reactive epoxides by β-oxidation. However, the predominant pathway of EET metabolism is conversion by soluble epoxide hydrolases (sEH) to the less active vicinal diols, dihydroxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-DHET).[12]

Recent studies are focusing on interaction between caveolins and signaling molecules; however, no evidence is available for the regulation of these proteins during ischemia-reperfusion. Moreover, decrease in EETs synthesis following caveolae disruption has been reported [16]; but relation between EETs and caveolins remains unexplored. In the present

study, we have determined the expression profile of Cav-1 and Cav-3 in mouse hearts, both before and after ischemia-reperfusion. In addition, our data suggest EETs prevent the loss of Cav-1, resulting in increased mitochondrial targeting and cardioprotection.

A1.2 Materials and Methods

A1.2.1 Immunohistochemical study

Animals were treated in accordance with the guidelines of Health Science Laboratory Animal Services, University of Alberta. C57BL6 male mice (aged 6-8 weeks) were purchased from Charles River Laboratories (Pointe Claire, PQ). Hearts were rapidly excised and perfused in Langendorff mode. In the pre-ischemic group, hearts were aerobically perfused for a 20min stabilization period; while in post-ischemic groups, hearts were subjected to 40min stabilization, 20min global no-flow ischemia, followed by 40min reperfusion.

Left ventricular myocardium (LVM) from both the groups was isolated and fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB, pH 7.2–7.4) overnight at 4°C. The fixed hearts were rinsed and cryoprotected in 30% sucrose in 0.1M PB overnight at 4°C. The cryoprotected hearts were embedded in Tissue-Tek® optimal cutting temperature (O.C.T.) compound and cryosectioned at 5µm thickness. Cryosections were attached to glass slides coated with 1.5% 3-aminopropyltriethoxysilane in acetone (Sigma Chemical Co., MO). To reduce artificial staining of non-specific proteins, 10% normal donkey serum (Calbiochem) was applied before applying primary antibody.

Primary antibodies against Cav-1 and Cav-3 were purchased from BD Transductions Laboratories (CA, USA) and Abcam Inc. (Cambridge,

MA), respectively. Cy3-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Labouratories (PA, USA) and Alexa488-conjugated donkey anti-rabbit IgG was from Invitrogen (OR, USA).

A1.2.2 Ultrastructural study

Two of each pre-IR and post-IR hearts were examined for the ultrastructural study. LVM was cut to 1mm^3 and placed in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.075M sodium cacodylate buffer (CB) for 2hr at 4°C to fix. The fixed LVM were briefly rinsed in 0.075M CB, post-fixed in 1% OsO4, dehydrated, and heat-polymerized with a mixture of Araldite50 and Embed812 for 48hr at 60°C. Ultra-thin sections of 70–90nm were cut and examined in a Philips-410 transmission electron microscope equipped with a charge-coupled device camera (MegView III) at 80kV.

A1.2.3 Immunoblot Analysis

Plasma-membrane, mitochondrial and cytosol fractions were prepared from frozen mouse hearts as described.[14] Protein (25μg) was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted as previously described.[14] Blots were probed with Cav-1 and Cav-3 to analyze protein expression changes in subcellular fractions. Prohibitin, GAPDH and Kir6.2 were used as loading controls for mitochondrial, cytosol and plasma-membrane fraction, respectively. Relative band intensities were assessed by densitometry using Image J (NIH, USA).

A1.2.4 Statistical Analysis

Values expressed as mean ± standard error of mean (SEM). Statistical significance was determined by the unpaired Student's t-test and one-way ANOVA. To determine whether significant difference exists between the groups, Newman-Keuls post-hoc test was performed. Values were considered significant if $p<0.05$.

A1.3 Results

A1.3.1 Immunohistochemical observations in cardiomyocytes and capillary endothelial cells

In the pre-ischemic group, Cav-1 was localized to both the plasma-membrane and cytoplasm of cardiomyocytes as well as at capillary endothelial cells (Fig. A1.1a). Cav-1 at the plasma-membrane and cytoplasm of the cardiomyocytes showed linear and punctate patterns, respectively. However, in the post-ischemic group, Cav-1 was absent at the plasma-membrane and cytoplasm of cardiomyocytes but not capillary endothelial cells (Fig A1.1b).

Interestingly, Cav-3 also localized at the plasma-membrane and cytoplasm of cardiomyocytes in both pre-ischemic and post-ischemic groups (Fig A1.1c). In the pre-ischemic group, Cav-3 showed a punctate pattern along the plasma-membrane and a network pattern at the cytoplasm of the cardiomyocytes. In the post-ischemic group, Cav-3 exhibited dense immunoreactivity along the plasma-membrane and poor network at the cytoplasm of the cardiomyocytes (Fig A1.1d).

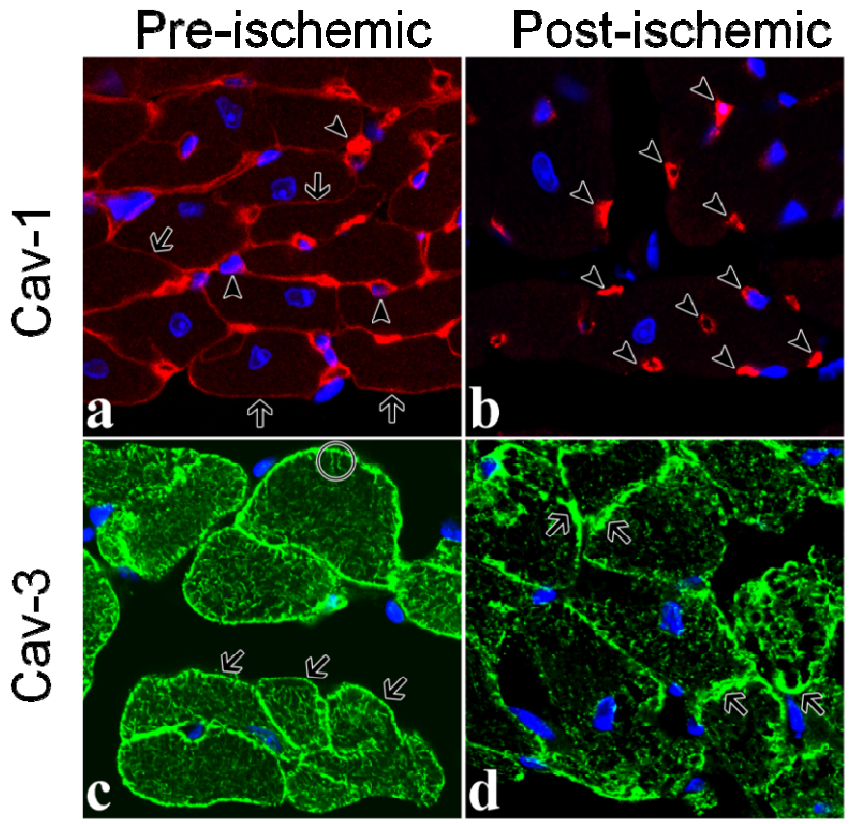

Figure A1.1: Immunolocalization of Cav-1 and Cav-3. *a*. Cav-1 in pre-ischemic group localized at both plasma-membrane (arrows) of the left ventricular myocytes and capillary endothelial cells (arrowheads). *b*. But Cav-1 in post-ischemic group lost their immune-reactivities at the plasma-membrane of the myocytes and revealed only at the capillary endothelial cells (arrowheads). *c*. Cav-3 in pre-ischemic group localized at plasma-membrane (arrows) and cytoplasm as net-like structures (circle). *d*. Interestingly, in post-ischemic group Cav-3 revealed its higher density than at plasma-membrane (arrows) in pre-ischemic group, and its net-like structures were damaged. In all images, nuclei are blue and scale bar is 10μm.

A1.3.2 Ultrastructural observations in cardiomyocytes

Caveolae, 50-100nm diameter flask-shaped invaginations, were observed either alongside or close to the plasma-membrane of pre-ischemic cardiomyocytes. Caveolae found along the plasma-membrane exhibit an open structure whereas caveolae located close to the plasma-membrane were closed depending on thin-section and their orientation (Fig. A1.2). In post-ischemic cardiomyocytes, no caveolae were observed either alongside or close to plasma-membrane (Fig. A1.2).At optimal longitudinal section, mitochondria and myofilaments, were revealed to have good distribution in the pre-ischemic group. Mitochondrial cristae were well preserved, and myofilaments showed good sarcomeric structure (Fig. A1.2). In the post-ischemic group, however, mitochondria lost the structural nature of their cristae, and myofilaments also lost constant electron density (Fig. A1.2). In addition, mitochondria were swollen according to quantitative analysis (data not shown).

Sacks of T-tubules in the cytoplasm of pre-ischemic group were located along z-lines and exhibited close contacts to mitochondria or sarcoplasmic reticulum (diad) (Fig. A1.2). In the post-ischemic group, the sacks of T-tubules were distinctly swollen. Distribution of glycogen particles in general were shown around mitochondria and myofilaments in the cytoplasm of pre-ischemic group, however in post-ischemic group glycogen particles were revealed as a bundle near plasma-membrane as well as around mitochondria and myofilaments (Fig. A1.2).

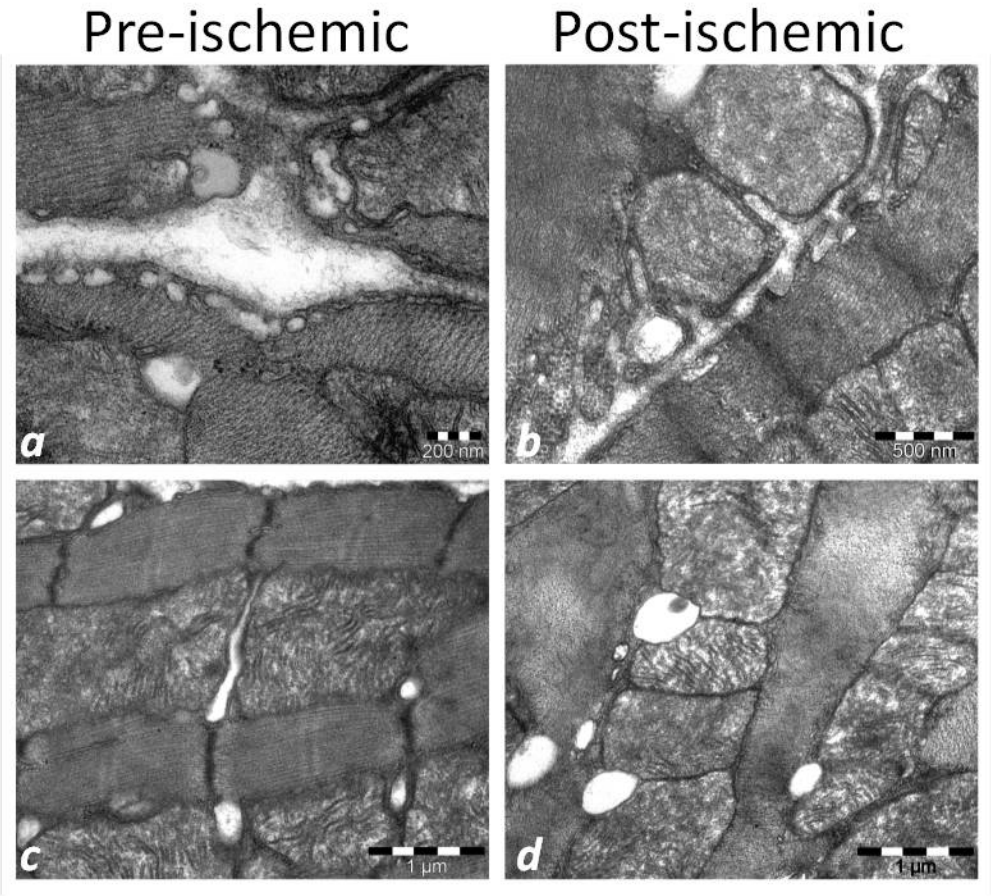

Figure A1.2: Ultrastructure onservations. Open and closed caveolae in pre-ischemic group showed along the plasma-membrane of the myocytes, but in post-ischemic group caveolae were absent. In pre-ischemic group cristae of mitochondria maintained their integrity, sacs of T-tubule were distributed along z-lines, and myofilaments were well preserved. However, in post-ischemic group, cristae of mitochondria lost or damaged their integrity, sacs of T-tubule were significantly swollen, and myofilaments lost their integrity and damaged. Distribution of glycogen particles was noticeable near plasma-membrane in post-ischemic group.

A1.3.3 Caveolins in EET mediated improved postischemic cardiac function

Consistent with our previous observations [15], sEH null and WT mice had normal baseline contractile function as measured by LVDP (Table A1). Furthermore, sEH null hearts had improved post-ischaemic LVDP function following 30min of global ischaemia and 40min reperfusion compared with WT hearts (LVDP: 21.8 vs. 45.7, respectively, $p<0.05$) (Table A1). Similarly, treatment of WT hearts with 11,12-EET improved post-ischaemic LVDP (21.8 vs. 52.8, $p<0.05$) (Table A1). To assess the effect of EETs on Cav-1 and Cav-3, we examined the expression of Cav-1 in plasma-membrane, mitochondrial and cytosolic fractions of WT, sEH KO, and 11,12-EET-treated WT hearts. In sEH KO and 11,12-EET-perfused hearts, significantly higher Cav-1 expression was observed in mitochondrial and plasma-membrane fractions (Fig A1.3 and Fig A1.4), while no difference was observed in Cav-3 expression at 40min of reperfusion (Fig A1.5), compared to WT hearts.

Table A1: Cardiac parameters.

	WT (n=16)	sEH null (n=9)	WT+11,12-EET (n=12)
Isolated Perfused Heart - Preischemic			
LVDP (cmH_2O) (Baseline)	118.4±7.9	119.5±11.3	116.7±7.2
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (Baseline)	3388±258	3182±517	3375±197
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (Baseline)	-2867±180	-2596±445	-2843±172
HR, perfused (beats/min) (Baseline)	322±16	347±26	354±11
Isolated Perfused Heart - Postischemic			
LVDP (cmH_2O) (R40)	21.8±2.4	45.7±8.0*	52.8±7.9*
Rate of contraction, dP/dt_{max} (cmH_2O/msec) (R40)	784±87	1271±182	1639±263*
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec) (R40)	-676±86	-1185±204*	-1377±221*
HR, perfused (beats/min) (R40)	303±16	296±17	333±11

Hemodynamic parameters were measured in isolated-perfused hearts. Values represent mean±SEM, * p<0.05 vs WT. LVDP, left ventricular pressure, HR, heart rate.

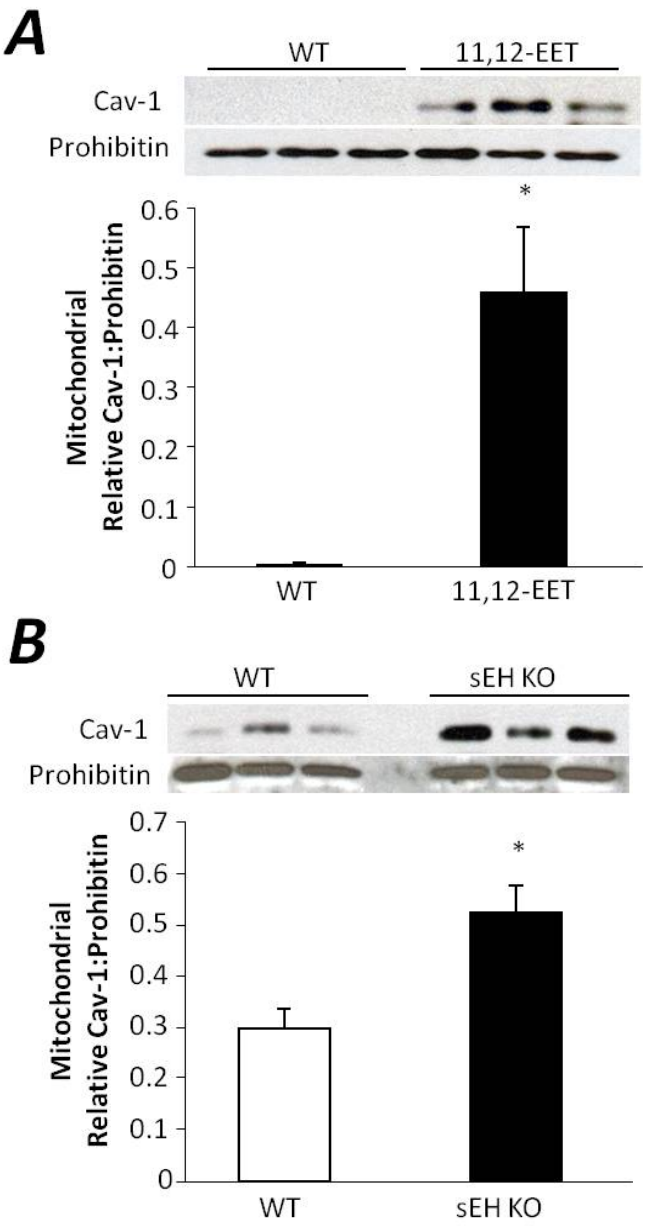

Figure A1.3: Cav-1 expression in mitochondria fractions of ischemic reperfused mouse hearts. *A* and *B*, Representative immunoblot and densitometry showing Cav-1 in mitochondrial fractions of sEH KO, 11,12-EET (1μM) or vehicle treated WT mouse hearts. Values represent mean ± SEM; n=5 per group; *, $p<0.05$ vs. WT.

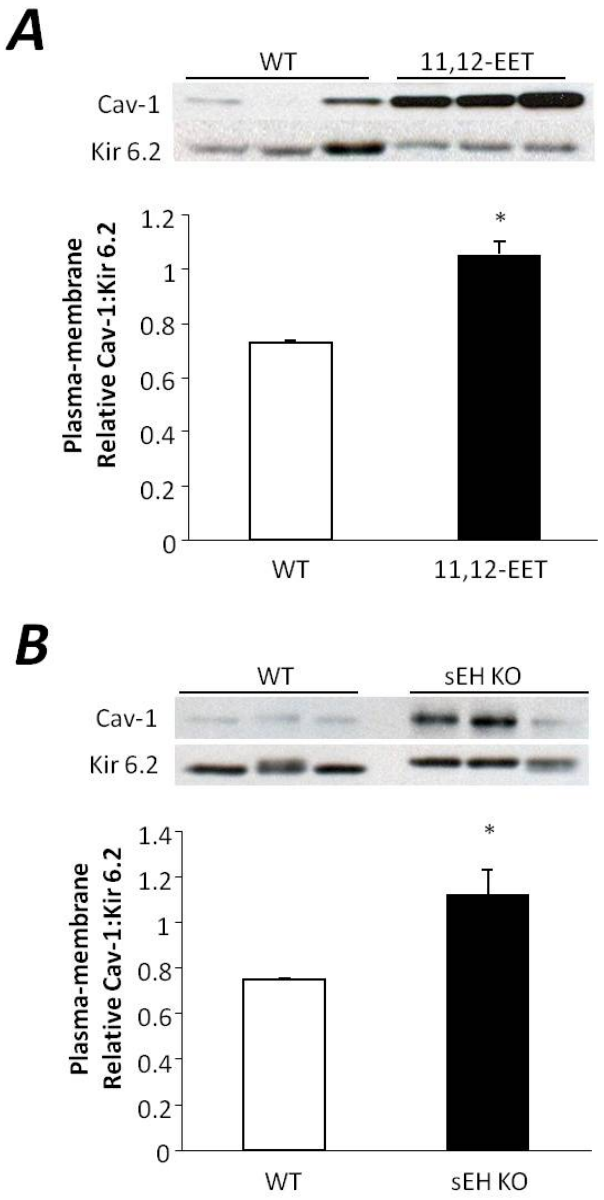

Figure A1.4: Cav-1 expression in plasma-membrane fractions of ischemic reperfused mouse hearts. *A* and *B*, Representative immunoblot and densitometry demonstrating Cav-1 in plasma-membrane fractions of sEH KO, 11,12-EET (1μM) or vehicle treated WT mouse hearts. Values represent mean ± SEM; n=6 per group; *, $p<0.05$ vs. WT.

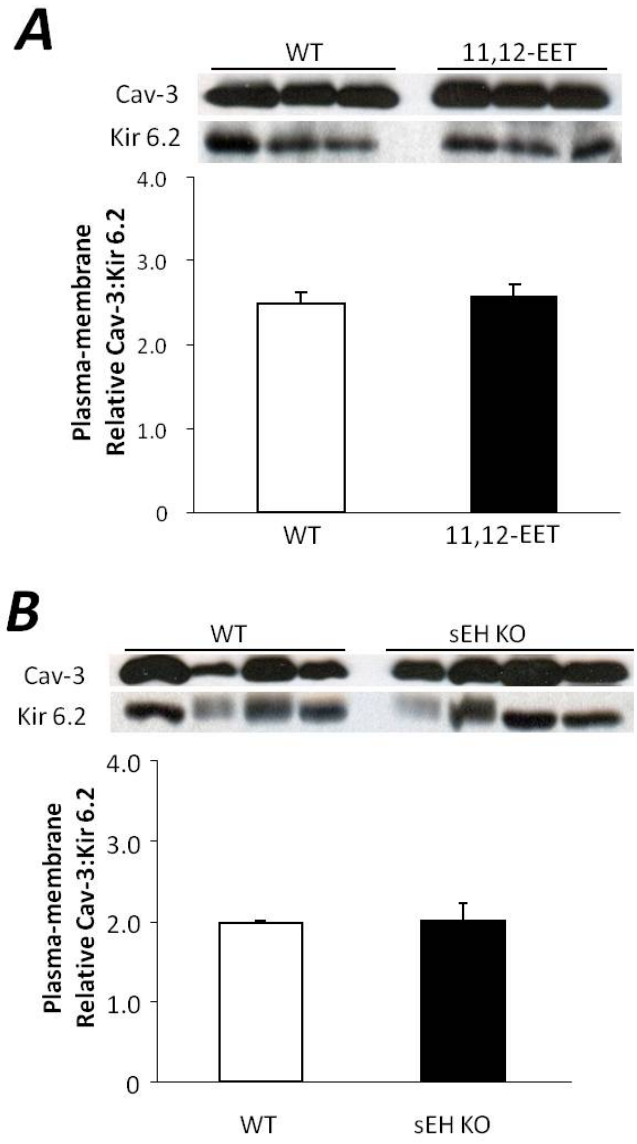

Figure A1.5: Cav-3 expression in plasma-membrane fractions of ischemic reperfused mouse hearts. *A* and *B*, Representative immunoblot and densitometry demonstrating Cav-3 in plasma-membrane fractions of sEH KO, 11,12-EET (1μM) or vehicle treated WT mouse hearts. Values represent mean ± SEM; n=6 per group; *, $p<0.05$ vs. WT.

A1.4 Discussion

In this paper, we report plasma-membrane and cytoplasmic localization of Cav-1 and Cav-3 prior to ischemia in left ventricular myocytes. While caveolae and Cav-1 disappeared from the plasma-membrane in response to global no-flow ischemia, Cav-3 remains unaffected. Moreover, mitochondria and myofilaments lost their structural integrity following ischemia-reperfusion. EETs prevented the loss of Cav-1 from plasma-membrane and increase mitochondrial targeting. Taken together, our data suggests that ischemic injury causes the loss of caveolae and Cav-1 from plasma-membrane, which may result in ultrastructural damage to mitochondria, and that EETs prevent the loss of Cav-1, increase mitochondrial targeting and thereby exert cardioprotective effects.

A growing body of evidence suggests that caveolae are essential to maintain cardiovascular homeostasis and to mediate cardioprotective signaling.[2, 10] In the present study, we report complete loss of caveolae from cardiomyocytes following ischemia-reperfusion. Caveolae deficient mice demonstrate dramatic decrease in left ventricular systolic function [17], which suggests that loss of caveolae may be responsible for low functional recovery in WT hearts following ischemia. Cav-1 and Cav-3 are structural proteins of caveolae, essential for formation of caveolae. While, targeted deletion of Cav-1 results in loss of caveolae from endothelial cells, Cav-3 deletion causes an absence of caveolae from

cardiomyocytes.[17] Interestingly, we observed complete loss of caveolae from plasma-membrane following ischemic injury, despite the presence of Cav-3. These observations indicate that only Cav-3 is not sufficient to maintain the morphology of caveolae structure in cardiomyocytes following ischemia. On the other hand, Cav-1 has been demonstrated to mediate cardioprotective signaling. Chow et al demonstrated that Cav-1 co-localizes with and negatively regulates matrix metalloproteinase-2 (MMP-2).[5] Detrimental effects of increased activity of intracellular MMP-2 have been shown to affect cardiac function following ischemia.[18-19] Chow et al also reported enhanced activity of MMP-2 in Cav-1 knock out mice.[5] Our data suggest the loss of Cav-1 following ischemic injury may result in a loss of the inhibitory effect of Cav-1 on MMP-2. Together, these data indicate that loss of Cav-1 and therefore increased activity of intracellular MMP-2 or decreased cardioprotective signaling may mediate detrimental effects of ischemia.

EETs have been linked to the activation of cell survival pathways, which converge onto the mitochondria thereby limiting mitochondrial damage and myocyte death caused by ischemia-reperfusion injury.[13-15] Katragadda et al have demonstrated that ischemia-reperfusion injury results in increased swelling and fragmentation of mitochondria that can be prevented by EETs.[13] However, the mechanism behind the protective effects on mitochondria remains unclear. Our data suggest that EETs prevent the loss of Cav-1 and increase mitochondrial targeting of Cav-1,

which may be the novel mechanism for EET-mediated cardioprotection.

Tubular aggregation has been demonstrated in the skeletal muscle of Cav-1 or Cav-2 knock out mice, whereas this effect was not observed in Cav-3 knock out mice.[20] Cav-2 knock out mice show mitochondrial swelling and tubular aggregation in skeletal muscle. The authors did not report mitochondrial structure in Cav-1 knock out mice; however, similarity in mitochondrial protein changes were observed in Cav-1 and Cav-2 knock out mice.[20] This indicates a similarity in structural damage to muscle cells in response to deletion of either Cav-1 or Cav-2. We found mitochondrial swelling and ultrastructure abnormalities in cardiac muscle cells following ischemia, which corresponds to the loss of Cav-1. Considering the fact that only Cav-1 and not Cav-2 is expressed in ventricular cardiomyocytes, indicates that Cav-1 is important for the normal structure of cardiomyocytes. Moreover, EETs prevent the loss of Cav-1, which further strengthens our hypothesis that Cav-1 plays a role in EET-mediated protection of mitochondria and cardiomyocytes following ischemia-reperfusion injury.

A1.5 Conclusion

We report the loss of caveolae and Cav-1 but not Cav-3 following ischemia-reperfusion, which suggest that Cav-3 alone is not sufficient to maintain plasma-membrane association of caveolae following ischemia. Ischemic injury affects mitochondria and myofilament ultrastructure

indicating that loss of Cav-1 may affect the morphology of cardiomyocytes following ischemia-reperfusion. Moreover, EETs prevent the loss of Cav-1, resulting in increased mitochondrial targeting, which is a novel mechanism of EET-mediated cardioprotection.

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