University of Alberta

Mechanisms by Which Arachidonic acid Metabolite, Epoxyeicosatrienoic acid Elicit Cardioprotection Against Ischemic Reperfusion Injury

by

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Dedication

I dedicate this thesis to my loving parents and grandparents.

ABSTRACT

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 epoxygenase metabolites of arachidonic acid that have cardioprotective properties but the exact mechanism(s) remains unknown. Evidence suggests phosphoinositide 3-kinase (PI3K) and plasma membraneATP-sensitive potassium channels (*pm*K_{ATP}) are important. However the role of the above two protective pathways and the corresponding intracellular cardioprotective mechanism involved is unknown. To investigate this, in the current study hearts from CYP2J2 transgenic mice or WT treated with either vehicle, EET, soluble epoxide hydrolase inhibitor or an EET analog (UA-8) were perfused in Langendorff mode for 40min of baseline and subjected to 20 or 30 min of global no-flow ischemia followed by 40 min of reperfusion. Hearts with elevated EETs had improved ventricular functional recovery and reduced injury compared to WT hearts following ischemia/ reperfusion. Blocking of *pm*K_{ATP} channel activity abolished these EET mediated protective effects. Similarly inhibition of PI3K activity, either with the pan specific PI3K inhibitor wortmannin or class-I, PI3Kα specific inhibitor, PI-103, abolished the EET-mediated protective effect, but other PI3K isoform specific inhibitors failed to block the functional recovery. In addition to the improved post-ischemic functional recovery, increased expression of p-Akt an PI3K downstream target, decreased calcineurin activity, Ca^{2+} activated enzyme, and decreased translocation of proapoptotic protein BAD to mitochondria were noted in EET elevated hearts. All these protective actions of EETs were abolished when *pm*K_{ATP} channel activity was inhibited however, increased expression of p-Akt was still observed in these hearts, suggesting PI3K pathway is still active. Further

in patch clamp experiments pre-treatment of myocytes with the PI3Kα inhibitor PI-103 significantly reduced the EET activation of *pm*K_{ATP} channels. Mechanistic studies using H9c2 cells demonstrate that EETs limit anoxia-reoxygenation triggered Ca^{2+} accumulation, decrease caspase-3 activity and maintain mitochondrial ΔΨm and decrease cell death compared to control. Both blocking of *pm*K_{ATP} channel and PI3Kα abolished EET mediated cytoprotection. Together our data suggest that EET-mediated cardioprotection involves activation of PI3Kα, upstream of *pm*K_{ATP}, which prevents Ca^{2+} overload and maintains mitochondrial function.

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LIST OF ABBREVIATIONS AND SYMBOLS:

AA	Arachidonic Acid
Akt/PKB	Protein kinase B
ANT	Adenine nucleotide translocatse
AMC	7-amino-4-methylcoumarin
APD	Action potential duration
Atr	Atracyloside
BK_{Ca}^{2+}	Calcium activated K^+ channels
CaN	Calcineurin
CAD	Coronary artery disease
CHD	Coronary heart disease
CM-H_2DCFDA	(5-(and-6)-chloromethyl– 2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester)
Cyc-D	Cyclophilin-D
CYP	Cytochrome P450 monooxygenase
DHET	Dihydroxyeicosatrienoic acids
DMEM	Dulbecco's modified Eagle medium
EET	Epoxyeicosatrienoic acids
EKG	Electrocardiogram
eNos	Endothelial NO synthase
ERK1/2	Mitogen activated protein kinases p42/p44
ETC	Electron transport chain
GIK	Glucose-insulin-potassium

GLIB	Glibenclamide
GSK3β	Glycogen Synthase Kinase-3
HETE	Hydroxyeicosatetraenoic acids
HPETE	Hydroperoxy eicosatetraenoic acids
HRP	Horseradish peroxide
IHD	Ischemic heart disease
IR injury	Ischemic–Reperfusion Injury
IPC	Ischemic Pre conditioning
IPost	Ischemic Post conditioning
LVDP	Left ventricular developed pressure
mitoK_{ATP}	Mitochondrial ATP sensitive potassium channels
mPTP	Mitochondrial permeable transition pore
MSPPOH	N-methylsulphonyl-6-(2-proparglyloxyphenyl) hexanamide
PCI	Percutaneous coronary intervention
PH	Pleckstrin homology domain
PiC	Mitochondrial phosphate carrier
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
pmK_{ATP}	Plasma membrane ATP-sensitive potassium channels
PtdIns	Phosphatidylinositols
PIP_2	Phosphatidylinositol 4,5 or 3,4 or 3,5 bisphosphate

PIP_3	Phosphatidylinositol 3, 4,5-Trisphosphate
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
QT	QT interval
QTc	Corrected QT interval
RPP	Rate-pressure product
ROS	Reactive oxygen species
sEH	Soluble epoxide hydrolase
ST	ST interval
STE	ST segment elevation
TMRE	Tetramethylrhodamine ethyl ester
TTC	2,3,5-triphenyltetrazolium chloride
UA-8	13-(3-propylureido)tridec-8-enoic acid
UA-7	13-(2-(butylamino)-2-oxoacetamido)tridec-8(Z)-enoic acid)
VDAC	Voltage gated anion channel
ΔΨm	Mitochondrial membrane potential
14,15-EEZE	14,15-epoxyeicosa-5(*Z*)-enoic acid
µg	Micro gram
µM	Micro Molar
nM	Nano Molar
I.P	Intra peritoneal

CHAPTER-I

INTRODUCTION

1.1. Overview

Cardiovascular disease (CVD) is a leading cause of illness, disability and death in both North America and developing countries.[1-3] While recent advances in research and health care have significantly improved outcomes, there still remains an increasing incidence and prevalence of death attributed to CVD. CVD is compromised of various conditions that involve the heart or blood vessels. Myocardial ischemia-reperfusion injury (IR) results from severe impairment of coronary blood supply producing a spectrum of clinical syndromes.[1-3, 4, 5] Damage to key organelles, like mitochondria that result in myocardial cell death is a critical contributing factor to morbidity and mortality in the pathogenesis of IR injury.[6-9] The role of mitochondria in the heart has progressed well beyond an ATP-generating organelle. Mitochodria are key regulators of both cell survival and cell death.[8, 9] Maintaining mitochondrial integrity and function is critical for the homeostasis of the cellular environment and organ function. Therefore, identifying novel therapeutic strategies that can target and protect the mitochondria from IR injury is an emerging field that will provide novel therapeutic targets.

Arachidonic acid (AA) is a polyunsaturated fatty acid bound esterified to cellular membranes, which can be released in response to stress stimuli such as ischemia.[10] Free AA is metabolised to numerous metabolites by cyclooxygenase, lipooxygenase and cytochrome P450 monooxygenase enzymes.[11, 12] AA metabolized by cytochrome P450 epoxygenases produces four regioisomeric epoxide metabolites (5,6-, 8,9-,11,12-and 14,15) called epoxyeicosatrienoic acids

(EETs).[11, 12] EETs are potent molecules that can activate intracellular signalling pathways in various tissues.[13-15] Accumulating evidence indicate EETs have important functional roles in the cardiovascular system. [16, 17] This thesis examines the cardioprotective role of EETs toward ischemia-reperfusion injury. Further it provides experimental data demonstrating that EETs decrease cellular injury and improve left ventricular function by limiting mitochondrial damage involving PI3Kα and plasma membrane ATP sensitive K+ channel (*pm*K_{ATP}) dependent mechanisms.

1.2. Cytochrome P450 Enzymes (CYP)

Metabolism is a process by which the structure of a compound is altered in the body which can result in either inactivation or bio-activation. In the body metabolism takes place in two phases and is catalyzed by specific enzymes, present in various organelles such as endoplasmic reticulum, cytosol and mitochondria. [18, 19] Phase I reactions involve the oxidation, reduction and hydrolysis of compounds that result in the addition or modification of the functional groups. Additional metabolism occurs via Phase II enzymes, such as methyltrasnferases, sulfotransferases, N-acetyltransferases, UDP-glucuronosyltransferases, glutathione S-transferases which conjugate moieties like sulfonate, glucuronic acid or glutathione, to further increase the water solubility and de-activate the element.[20, 21]

Cytochrome P450 (CYP) mono-oxygenases are a super family of heme containing enzymes that catalyze phase I oxidation reactions.[20-22] These enzymes are present in protozoa to humans and are classified into different families and

subfamilies based on the amino acid sequence.[23, 24] Proteins sharing >40% amino acid identity are grouped under one single family, by an Arabic numeral next to the abbreviation (i.e., CYP1). Members within the same family sharing >55% amino acid identity are grouped under the same subfamilies, represented by an alphabet (i.e., CYP1A) and the individual gene (i.e., *CYP1A1*). [Mouse nomenclature are represented by lower case (i.e., Cyp1a1). [21, 23-25]] CYP enzymes are important and are involved in both exogenous and endogenous metabolism. CYP substrates include metabolic intermediates such as lipids, steroidal hormones, xenobiotics such as drugs and other toxic chemicals, which are known to play vital roles in the physiological function of cells and tissues. [26, 18, 20, 21, 23, 25]

1.2.1 Catalytic Cycle of Cytochrome P450 Enzymes

CYP enzymes exist in a mixed function oxidase system comprised of cytochrome P450, FMN/FAD-NADPH dependent cytochrome P450 reductase, and phospholipids.[25] The catalytic cycle begins when a substrate binds to the ferric (Fe^{3+}) form of the enzyme. An electron is then transferred from NADPH, by cytochrome P450 reductase, reducing the heme iron from the ferric (Fe^{3+}) to the ferrous state (Fe^{2+}), which then binds to molecular oxygen (O_2). A second electron is then transferred to this complex by cytochrome P450 reductase resulting in peroxide (OOH) complex. Being a very unstable intermediate, one atom of O_2 is released as H_2O resulting in formation of $Fe^{3+}O$ complex. The Fe^{3+} O complex transfers one molecule of O_2 to substrate, regenerating the Fe^{3+} oxidized form of the enzyme, completing the cycle.[21, 25]

1.2.2 Cardiac Expression of Cytochrome P450 Enzymes

While CYP enzymes are predominantly expressed in liver, significant levels are found in various extra hepatic tissues such as lung, kidney, gastrointestinal tract, and heart. [27-29] The metabolic and drug clearance capacity in extra-hepatic organs is low and/or relatively unknown. However, evidence demonstrates that extra hepatic CYP enzymes play an important role in the metabolism of endogenous and exogenous substrates, important to tissue function and homeostasis.[27]

CYP enzymes found expressed in the heart include members of the CYP1, CYP2, CYP3, CYP4 and CYP11 families. [26-28, 30, 31, 32] In the human heart constitutive expression of CYP1 family members is very low but specific isozymes are inducible under certain conditions. CYP1A1 mRNA is expressed in the endothelial cells of left ventricle in healthy human heart, whereas in patients with cardiac disease, expression was detected in right the ventricle, right atrium and aorta.[31, 33, 34] In contrast, expression of CYP1A2 mRNA is not detectable in healthy conditions but increased expression occurs under pathological conditions.[31, 33] Studies demonstrating the expression levels CYP3 family members in the human heart have been mostly inconsistent and remain unclear.[31, 33] In contrast to the CYP3 family, CYP4 family members are found to be highly expressed in the heart. For example, CYP4A1, CYP4A2, and CYP4F expression were detected in dog and rat heart tissue and CYP4F12 has been detected in human heart. CYP4B1 mRNA was also detected but only in the right ventricle in the patients with dilated cardiomyopathy.[31, 33, 34] Enzymes from the CYP11

family are found in the heart, such as CYP11A mRNA in normal and failing heart and CYP11B mRNA in the failing heart. [31-34]

Important to the current thesis is the cardiac expression and function of members from the CYP2 family which have epoxygenase activity. CYP2 enzymes catalyze the epoxygenation of arachidonic acid, leading to formation of active secondary metabolites. [16, 35-38] CYP2C isoforms, CYP2C8 and CYP2C9 are found to be expressed in the heart, vasculature and smooth muscle cells. In addition, CYP2J isoforms, notably human CYP2J2, rat CYP2J3 and mouse cyp2j5, are expressed in cardiomyocytes at levels 1,000 times higher in normal human hearts and to smaller extent in the endothelial cells.[16, 32, 37, 38] Cardiac expression of other CYP2 isozymes such as CYP2D6 and CYP2E1 have been observed in both normal and diseased human hearts, however limited information is available regarding these enzymes.

Overall, the current level of knowledge regarding the expression of various CYP enzymes in the cardiovascular system depends on the condition of the heart. Importantly, the specific role and function of each CYP isozyme within the heart remains unclear.

1.3. Arachidonic Acid (AA) and its Metabolites

AA ($C_{20}H_{32}O_2$) is a polyunsaturated fatty acid composed of a 20-carbon chain with four *cis* double bonds; the first double bond is located at the sixth carbon from the omega end.[39] AA is synthesised from linoleic acid which is a ω-6 polyunsaturated fatty acid, an essential fatty acid found in vegetable oil, safflower, sunflower, corn oil and poppy seed. Linoleic acid is converted to AA

through a multistep process. Briefly, linoleic acid is metabolized by Δ^6desaturase, converting it into gamma linolenic acid. Next, the carbon chain is elongated by elongase generating dihomo-gamma-linolenic acid, which is further metabolized by Δ^5desaturase producing arachidonic acid. In addition to synthesis from the linoleic acid, AA can be directly obtained from dietary sources such as eggs, meat and dairy products. AA is found esterified in membranes to the SN-2 position of phospholipids like phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) of most cells and organelles within the body. In response to various hormones or stress stimuli, such as ischemia AA can be released into the cell by the calcium activated phospholipase A_2 (PLA_2). PLA_2 hydrolyzes the ester linkage between the phospholipids releasing free AA.[10, 37, 40, 41] Free AA can be further metabolized by cyclooxygenases, lipooxygenases and cytochrome P450 monoooxygenases to multiple products that mediate various adaptive or inflammatory signals within the body (Fig.1-1).

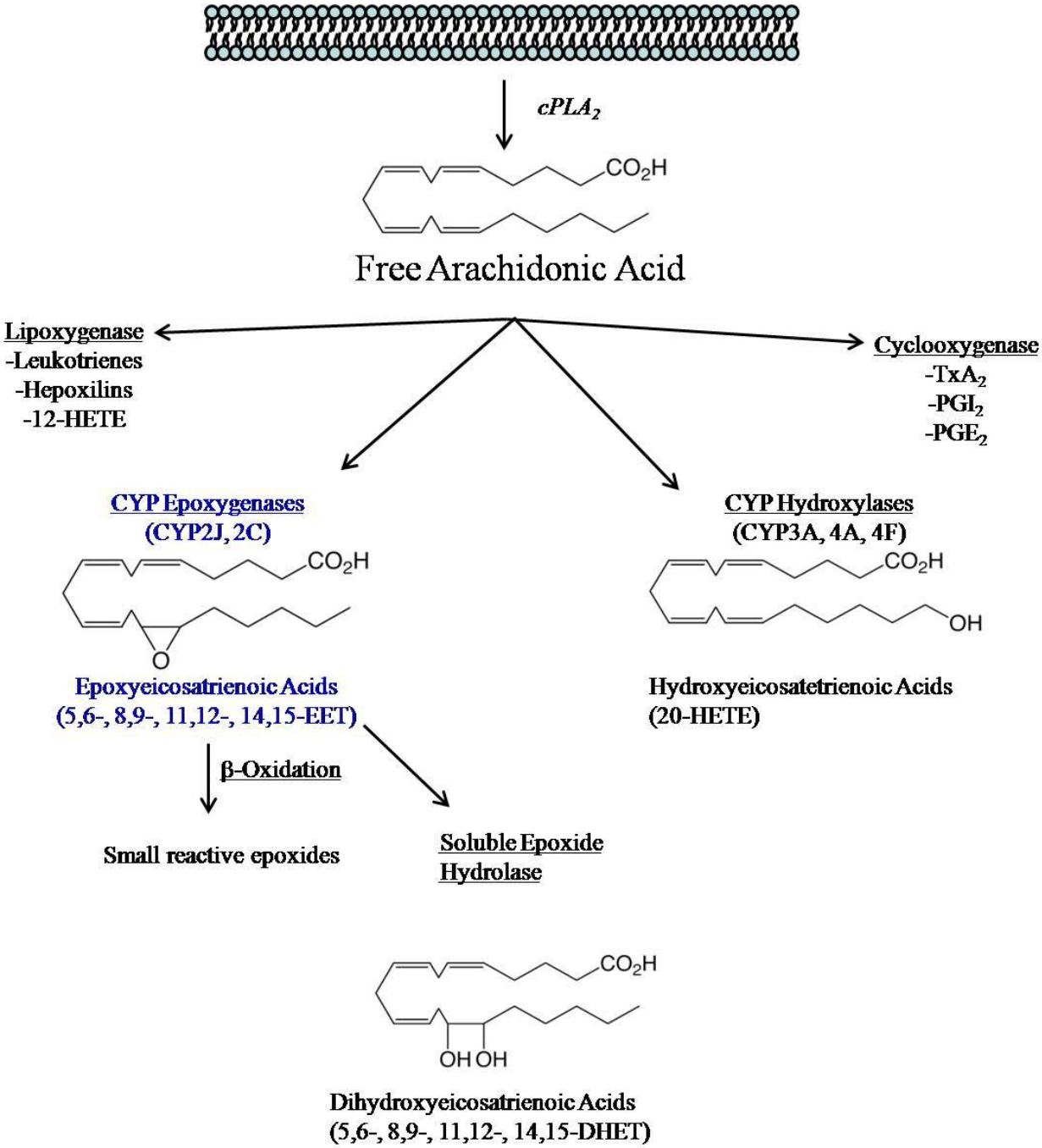

Fig.1-1 Arachidonic acid metabolism (Seubert *et al*, Prostaglandins Other Lipid Mediat.2007Jan;82(1-4):50-9)

Lipoxygenases (LOX) are a class of enzymes that metabolize AA to bioactive metabolites such as leukotrienes and lipoxins. There are different LOX isozymes (5-, 8-, 12-, and 15-LOX) which metabolize AA to the corresponding hydroperoxy eicosatetraenoic acids (5-, 8-, 12-, 15-HPETE) by dioxygenation. Reduction of these metabolites leads to production of corresponding hydroxyl derivatives (5-, 8-, 12-, 15-HETE). HPETE metabolites can be metabolised further to leukotrienes and lipoxins. [11, 12, 42] Cyclooxygenases are another well-characterized class of enzymes that metabolize AA to active metabolites such as prostaglandins (PG) and thromboxanes (TX). [11, 12, 42] There are two predominant COX isozymes, COX-1 and COX-2; both enzymes produce similar products but significantly differ in expression patterns and tissue localization. COX-1 is constitutively expressed while COX-2 expression is inducible by various stimuli.

AA is also metabolised by a third, less characterized pathway; cytochrome P450 (CYP) monooxygenase enzymes which produce metabolites such as 20-hydroxyeicosatetraenoic acids (20-HETEs) and epoxyeicosatrienoic acids (EETs). 20-HETE and EETs have different important cardiovascular effects, For example, 20-HETE causes vasoconstriction and produces many detrimental effects in the heart following ischemic-reperfusion injury while EETs are cardioprotective. CYP 3A, CYP 4A and CYP 4F families catalyze the hydroxylation of AA carbon 20 to generate 20-HETE. [37, 39, 43]

EETs are produced by CYP epoxygenases from different families with isoforms expressed in various organs such as lung, liver, heart, gastrointestinal tract, kidney, and vasculature. [37, 39, 43] Cardiac CYP2 families are the predominant

epoxygenases involved in AA metabolism, in particular CYP2C8, CYP2C9 and CYP2J2. [12, 31, 38, 42] These epoxygenases metabolize AA by replacing one of the four double bonds on arachidonic acid with an epoxide group leading to the formation of secondary metabolite, EETs. Based on the position of the double bond the corresponding EET regioisomers, 5,6-, 8,9-, 11,12- and 14,15- EET, are generated. Each regioisomer is produced as a mixture of the S/R and R/S enantiomers. CYP isoforms produce these in varying amounts, for example, CYP2C8 produces 80% 14(R), 15(S)-EET and 81% 11(R), 12(S)-EET entantiomers whereas CYP2C9 produces these enantiomers as 60% 14(R), 15(S)-EET and 30-40% 11(R), 12(S)-EET. Similarly CYP2J2 produces 47% 8(R), 9(S)-EET and 53% 8(S), 9(R)-EET, 60% 14(R), 15(S)-EET and 30-40% of 11(R), 12(S)-EET enatiomers. [36, 37, 38]

EETs are biologically active metabolites involved in many intracellular signalling pathways in the cardiovascular system. Interestingly, EETs produce a very diverse array of effects through both autocrine and paracrine actions. All EET regioisomers have demonstratable effects, but the potency depends upon the organ system and specific enantiomer. [36, 37, 38] For example, in the vasculature 11(R),12(S)-EET is more active than 11(S), 12(R)-EET in rendering vasodilation. In contrast 14(S),15(R)-EET is more potent than 14(R),15(S)-EET in eliciting a hyperpolarezation of vascular smooth muscle through activation of Ca^{2+}-sensitive K^+ channels (BKca) and ATP-sensitive potassium channels (K_{ATP} channels) resulting in vasodilatation of coronary vessels, while 5,6-EET and 8,9-EET produce effect via TRPV4 channels.[12, 35, 36, 44, 45, 46, 47, 48] 11-12-EET has been

shown to produce anti-inflammatory and anti-thrombolytic properties within the vasculature by inhibiting IKK-mediated phosphorylation of IkBα and increasing the tissue plasminogen gene expression. [13, 14, 12, 35, 36, 44, 45, 46, 47, 48] Other EET-mediated effects include, 8,9-EET and 11,12-EET triggered angiogenesis, cell proliferation and anti-apoptotic properties occurring through activation of p38 MAPK whereas 5,6-EET and 11,12-EET produce this effect through activation of PI3K. [49, 50] There is a growing body of experimental evidence in animal models demonstrating how EETs can regulate cardiac vascular diseases such as IR injury, hypertension and prevents progression of atherosclerotic plaque formation (Fig.1-2).[36, 51, 52]

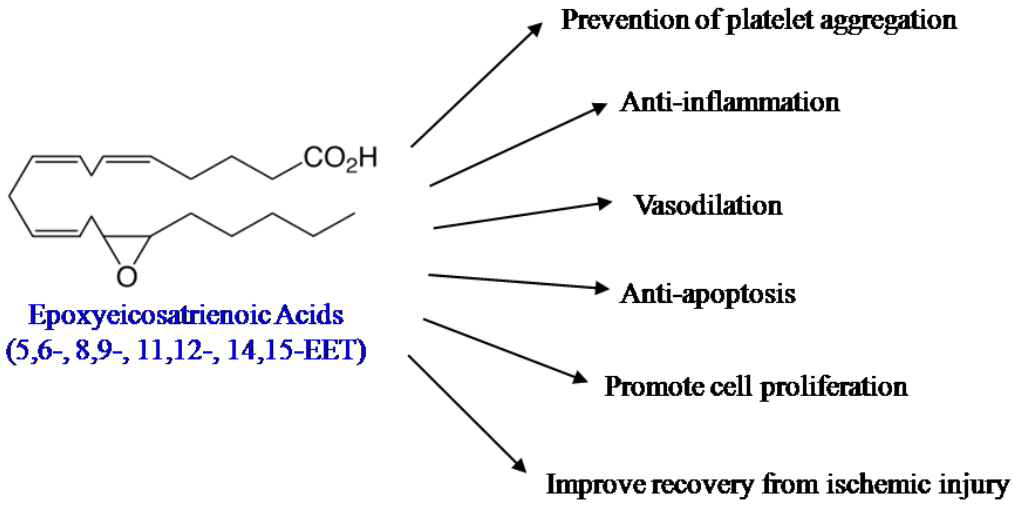

Fig.1-2 Functions of epoxyeicosatrienoic acids

EETs can be found esterified to phospholipids in cellular membranes and are released following stress or further metabolized to inactive metabolites by β-oxidation, auto-oxidation and chain elongation. [35, 36] The predominant route of EET metabolism is hydroxylation to dihydroxyeicosatrienoic acids (5, 6-, 8, 9-, 11, 12-, and 14, 15-DHET) by soluble epoxide hydrolase (sEH).[53-55] Two major

epoxide hydrolases are found in mammalian tissues, the microsomal epoxide hydrolase (mEH) and the soluble epoxide hydrolase (sEH or *Ephx2*).[54] Studies have demonstrated that sEH is the main enzyme involved in the *in vivo* hydrolysis of EETs. [17, 54] sEH is a bi-functional enzyme with C-terminus hydrolase activity and N-terminus phosphatase activity. [53-55] While the functional effect of the phosphatase is not well known, evidence suggests that the hydrolase activity of the sEH enzyme is responsiable for the hydrolysis of EETs. This has been demonstrated in two different models, one a genetic modification approach by targeted deletion of the *Ephx2* gene, and two sEH enzyme pharmacological inhibitors that block the hydrolase activity of the enzyme, suggesting that sEH will hydrolyze EETs. [12, 17]

Relevant to the current thesis, there is a growing amount of evidence demonstrating the cardioprotective effects of EETs toward ischemia-reperfusion injury. Various animal models such as transgenic mice with over expression of human CYP2J2 or mice, rat, rabbits and dogs treated with different synthetic EETs regiosiomers, results have demonstrated EETs can improve cardiac contractile function and reduce infarct size.[16, 56-59] Moreover, inhibition of EET production or perfusion with an EET antagonist, 14,15-EEZE, abolishes its protective effect. [16, 17] Indeed, the inhibition of sEH enzyme either by pharmacological agents such as CDU, AUDA, AUD-BE, *t*AUCB or genetic alteration, results in elevated cellular EET levels producing significant cardioprotective actions.[17, 36, 52, 60, 61] Evidence from human epidemiological studies have identified polymorphisms in *CYP2J2* and *Ephx2* which have been

associated with increased risk of cardiovascular disease.[62-65] These studies provide strong evidence that CYP derived AA metabolites, EETs, are cardioprotective. As such, maintaining EET levels in the body will help in alleviating ischemic-reperfusion injury under both clinical and pathological conditions.

1.4 Mitochondria

Mitochondria are important organelles that not only provide the energy required for a cell, but have a key role in regulating cell survival and death.[66] Structurally, mitochondria contain two membranes composed of phospholipids and proteins, the outer membrane is smooth whereas the inner membrane is highly convoluted, forming folds called cristae.[66] Cristae increase the inner membrane surface area which harbor proteins, such as ATP synthase, involved in energy production in the form of ATP, which is the primary energy source of a cell.[66] Considering the heart has a very high demand for energy, there is a need for a continuous flow of ATP to maintain normal cardiac function. Importantly, mitochondria occupy more than 30% of the cardiac volume and provide energy in the form of ATP required by the heart to pump blood throughout the body.[67, 68]

Glycolysis, a metabolic pathway, occurs in the cytosol under both aerobic and anaerobic conditions, resulting in oxidation of glucose to pyruvate.[66, 69] Under aerobic conditions the pyruvate formed is converted to acetyl Co-A which can enter the citric acid cycle to produce ATP.[66] Under anaerobic conditions pyruvate is converted into lactate. The production of ATP from oxidation of glucose is higher during aerobic respiration compared to anaerobic respiration. [66, 69] Under aerobic conditions pyruvate is transported into the mitochondria where it is

oxidized and combined with coenzyme A to form CO_2, acetyl-CoA and NADH. This acetyl-CoA is also formed from fatty acids which are transported into the mitochondria through mitochondrial β-oxidation under normal aerobic conditions. Acetyl-CoA formed is the primary substrate for the citric acid cycle, also known as the tricarboxylic acid cycle or Krebs cycle.[66, 69] The enzymes in the citric acid cycle convert acetyl-CoA into CO_2, during the process three molecules of NAD and one molecule of FAD and a molecule of GDP are converted into NADH, $FADH_2$ and GTP. NADH and $FADH_2$ are the primary source of electrons for the electron transport chain (ETC).[66, 69] The ETC is comprised of a series of enzymes and proteins that shuttle electrons between donors and acceptors to pump H^+ ions (protons) across the inner membrane to generate a proton motive force.[70] The resulting electrochemical gradient is used to generate energy in the form of ATP.[71] Electrons from NADH and $FADH_2$ are passed to more electronegative acceptors (Complex-I or II) through electron transfer proteins which in turn donate the electrons to another acceptor, (complex-III and IV). The process is continued until electrons are passed to oxygen, the most electronegative and terminal electron acceptor in the chain.[66, 69] As electrons are passed between donors and acceptors, the energy released is used to pump H^+ ions into the inter-membrane space generating a proton gradient and electrical potential ($\Delta\Psi$) (proton motive force) across the mitochondrial membrane. Under normal conditions H^+ moves back through the F_OF_1 ATP synthase complex into the mitochondrial matrix via the F_O component. [72] Energy released during the reflux, is used by the F_1 component of the F_OF_1 ATP synthase complex to synthesis ATP.

[66, 72] Out of the total energy generated by the mitochondria more than 70% of the ATP is used by the contractile apparatus of the heart to generate contractile force and the rest is used up by the different ion channels and pumps which help in regulating ion balance or homeostasis inside the cell (Fig.1-3).

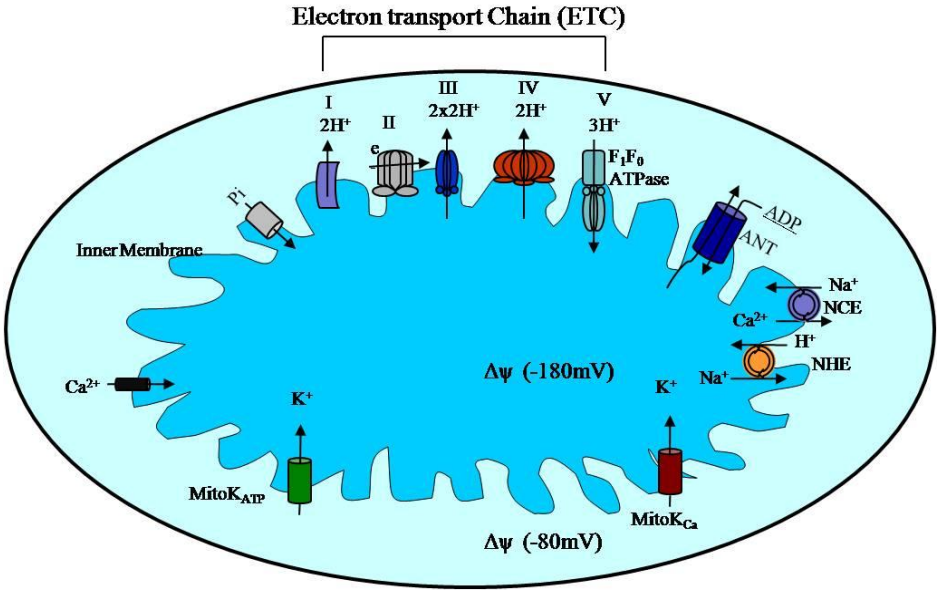

Fig 1-3 Schematic representing structures and energetics of mitochondria

In addition to the generation of energy, mitochondria under pathological conditions can initiate and regulate cell death processes. Under stress conditions such as oxidative stress or cellular damage, mitochondria switch from energy production to cell death. This initiates specific processes that mediate cell death, for example by releasing pro-apoptotic proteins such as cytochrome *c*, apoptosis inducing factor and Smac/Diablo. In the heart this switch has been shown to have very severe effects, decreasing cardiac efficiency and increasing overall cell death. It has been suggested that IR injury causes severe damage to these organelles resulting in cardiac cellular dysfunction. [73, 74]

1.5 Ischemic–Reperfusion Injury

1.5.1 Ischemic Injury

Ischemic injury results from an imbalance between oxygen demand and oxygen supply due to interruption in blood flow. It has been long established that this decrease in blood supply is due to a consequence of an underlying disease like atherosclerosis, which causes stenosis or narrowing of arteries. As a result of this cellular metabolic profile changes resulting in reduction of intracellular ATP production, disturbances in ion homeostasis and accumulation of catabolites and cell death.

Pathophysiology

Significant changes are known to be initiated within seconds of the onset of ischemia. The extent of injury depends on the duration between interruption of blood flow and reperfusion, that is these effects are initially reversible, if blood flow is restored; but if oxygen is deprived for an extended period of time it becomes more severe, leading to irreversible damage. The series of events that takes place inside the cell during ischemia can be generally characterized as follows. Reduced oxygen supply during ischemia inhibits the respiratory chain and oxidative phosphorylation, there by, limiting production of ATP. Subsequently, there is a severe drop in the ATP/ADP ratio and increase in the Pi (phosphate). To maintain energy production and cellular function the cell switches from aerobic fatty acid/ glucose oxidation to anaerobic respiration, glycolysis.[4, 75] The switch to glycolysis results in an increased production of lactic acid and H^+ ions leading to drop in the intracellular pH. Accumulation of NADH and H^+ ions

eventually inhibits glyceraldehyde phosphate dehydrogenase halting glycolysis.[4, 75]

The increase in acidic conditions significantly alters intracellular Na^+ concentrations resulting in increased Na^+ influx via the Na^+/H^+ antiporter. Build up of Na^+ ions inside the cell stems from the drop in ATP levels which impedes proper function of ATP-dependent ion pumps such as Na^+/K^+ ATPase, impacting ion homeostasis. [5, 76-79] In addition, cytosolic Ca^{2+} concentrations also significantly increase during ischemia. This results from Ca^{2+} influx via the reverse mode operation of Na^+/Ca^{2+} exchanger, due to reduction of Ca^{2+} uptake into the sarcoplasmic reticulum and Ca^{2+} efflux by Ca^{2+}ATPase pump activity.[5, 76-79] Mitochondria normally act to buffer cytosolic Ca^{2+} overload; however, excessive Ca^{2+} accumulation leads to mitochondria damage. Elevated Ca^{2+} levels will activate many proteases and phospholipase enzymes, which begin to breakdown cellular and mitochondrial components leading to organelle dysfunction and release of apoptotic factors, such as cytochrome *c*. Overall, there is an increase in intracellular ionic and catabolites levels during ischemia that leads to increased intracellular osmolarity, membrane depolarization and membrane permeability (Fig1-4). It has been demonstrated that cytoplasmic osmolarity increases by more than 100m Osm after 45-60 min ischemia *in vivo*.[80]

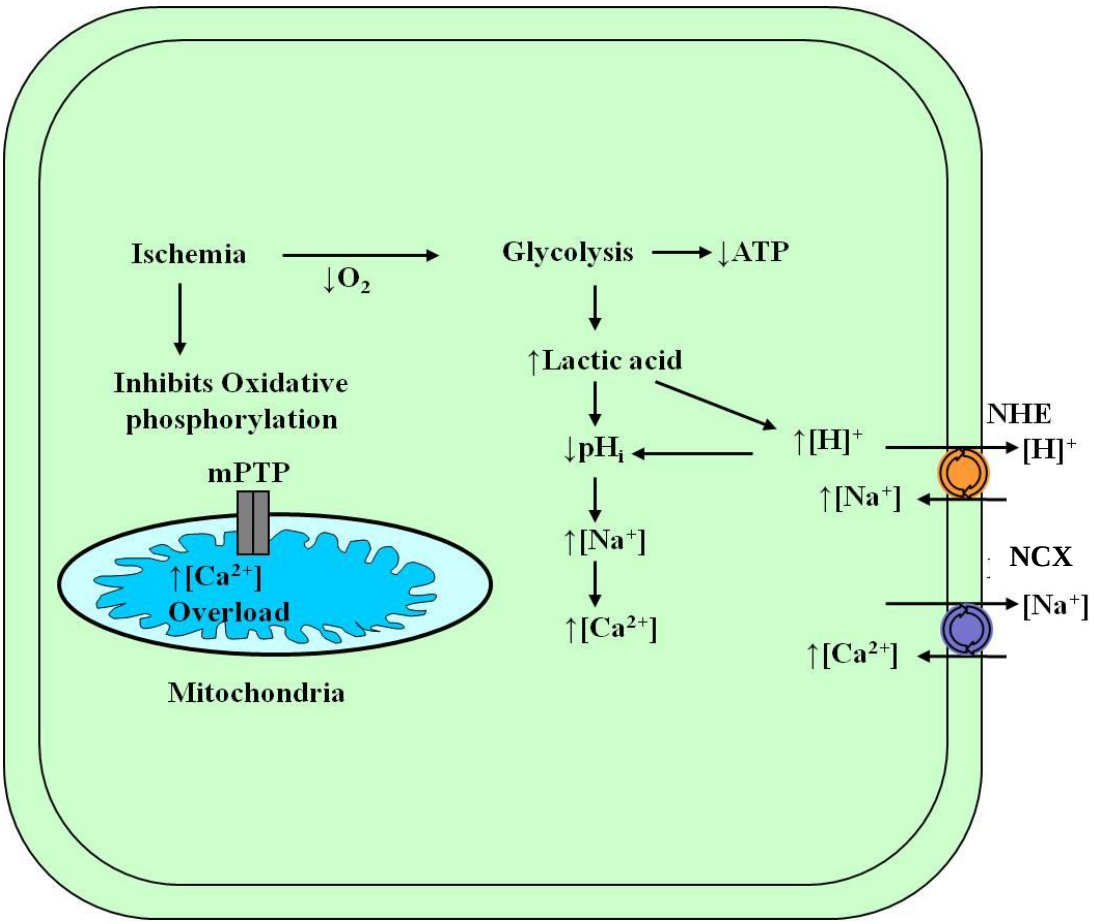

Fig1-4 Schematic representation of metabolic and ionic concentration changes inside the cell following ischemic injury.

1.5.2 Reperfusion Injury

In 1960`s Jennings and Reimer suggested reperfusion of blood flow to ischemic regions is important to salvage or reduce tissue injury.[81] However, reperfusion can lead to further complications and worsen the injury initiated by ischemia. Oxygen radicals and Ca^{2+} overload are thought to be the major players responsible for reperfusion injury.

Pathophysiology

Reperfusion will lead to restoration of extracellular pH levels, which trigger exchange of cytosolic H^+ for Na^+ by the NHE pump resulting in a further increase in intracellular Na^+ concentrations.[5, 77, 78] The rise in intracellular Na^+ concentrations are controlled by the Na^+/K^+ ATPase pump and the reverse mode of NCX. However, during the initial period of reperfusion, ATP levels remain relatively low, as the excess Na^+ is handled mostly by NCX which results in a rise in cytosolic Ca^{2+}.[5, 77, 78] Consequently, the heart is susceptible to hypercontraction, arrhythmias and increased osmolarity during reperfusion.[76] In addition during reperfusion while glycolytic rates remain elevated fatty acid oxidation still dominates as a source of oxidative energy production. This fatty acid oxidation further leads to more Ca^{2+} accumulation.

Difficulty during the reperfusion period can arise from consequences resulting from dysfunctional mitochondria as result of the previous ischemia period.[80, 82] Re-introduction of oxygen initiates mitochondrial re-energization and reactivation of the ETC, which restorates mitochondrial membrane potential and increases ATP production. However, the sudden restoration causes further Ca^{2+} influx from the cyotsol into the mitochondrial matrix leading to mitochondrial Ca^{2+} overload and subsequent disruption of oxidative phosphorylation leading to generation of ROS.[80, 82] These events ultimately trigger opening of mitochondrial permeability transition pore (mPTP) leading to swelling of mitochondria, alterations in mitochondrial membrane potential and release of pro-apoptotic proteins (Fig 1-5).

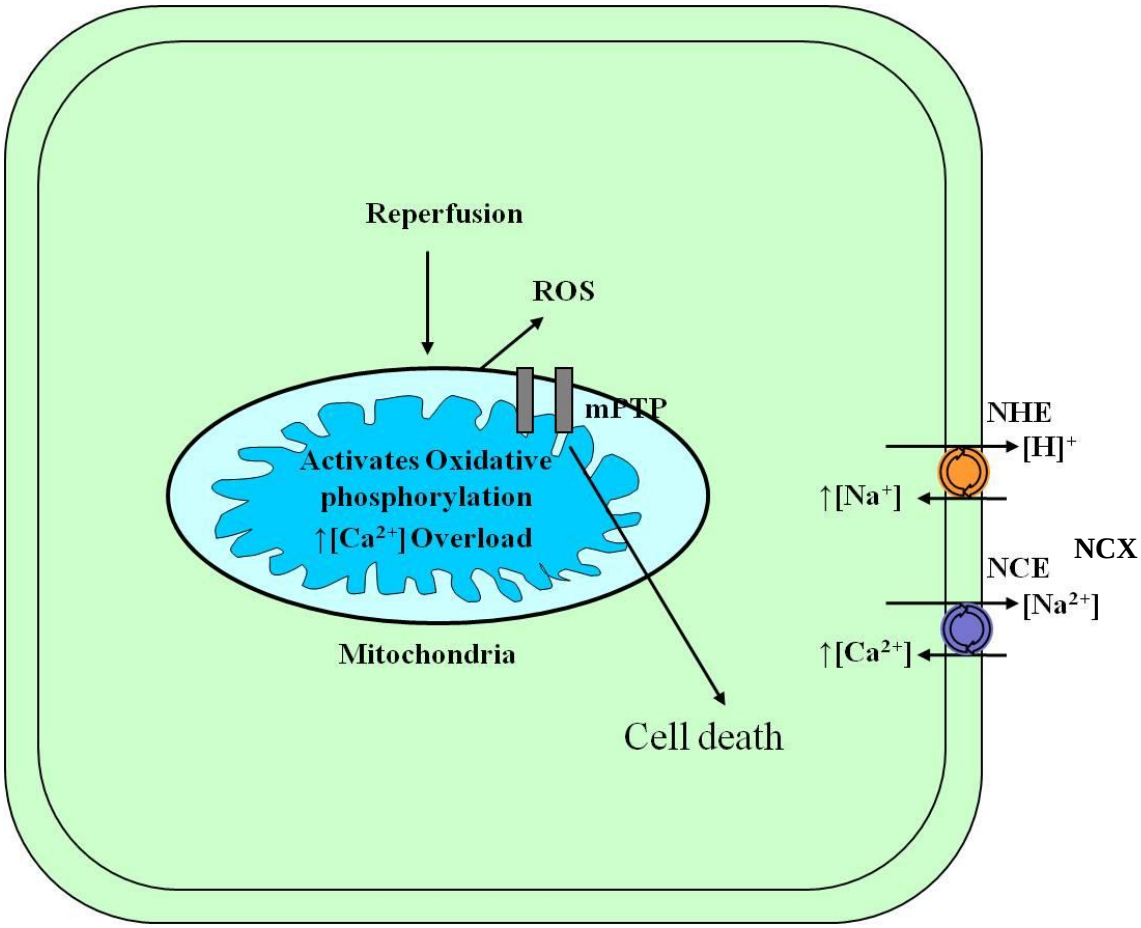

Fig 1-5 Schematic representation of changes seen inside the cell following reperfusion injury

Mitochondrial permeablility transition pore (mPTP)

mPTP is a multiprotein complex that spans both mitochondrial membranes. Currently, the molecular composition and structure is poorly defined, however evidence suggests it is composed of proteins found on the outer membrane, such as voltage gated anion channel (VDAC), and proteins found on the inner membrane, such as adenine nucleotide translocatse (ANT), cyclophilin-D and mitochondrial phosphate carrier (PiC).[83, 84,73, 74]

Opening of mPTP allows free passage of molecules >1.5 kDa which can initiate adverse effects like alterations to mitochondrial osmotic pressure, uncoupling of oxidative phosphorylation and release of apoptotic factors such as cytochrome *c* and Smac/Diablo.[73, 74, 83-86] Under normal physiological conditions mPTP opens briefly and rapidly closes, however, following significant cellular stresss, such as IR injury, it will remain open.[73, 74, 83-86] Evidence indicates that IR injury intiates opening of mPTP by triggering a conformational change in the membrane proteins set off by Ca^{2+} and Cyp-D binding to ANT in its *C* confirmation, pore opening mode. In contrast, the M conformation, the pore closing mode, binding of Cyp-D has the least effect in inducing pore opening.[83, 84, 87] ROS can also mediate the opening of the mPTP but the mechanism is not known.[84, 87] Several pro-apoptotic molecules from the Bcl-2 family proteins, like Bax, BAD and Bid, trigger mPTP following IR injury. IR injury initiates Bcl-2 proteins to translocate to mitochondria and permeabilize the outer membrane of the mitochondria leading to depolarisation of its membrane potential, stimulating opening of mPTP and allowing release of pro-apoptotic inter membrane proteins like cytochrome *c* into the cytosol. [88-91] Inhibition of mPTP during reperfusion with pharmacological agents such as cyclosporine-A will prevent Ca^{2+} overload or antioxidants will reduce cardiac injury (Fig.1-5).[83, 84, 86]

1.6 Cardioprotection

Cardioprotection is a general term which refers to the mechanisms and means that lead to the preservation of the myocardium by reducing or preventing damage associated with acute myocardial infarction.[92] Over the past 35 years a

number of experimental interventions have been studied using both pharmacological and non-pharmacological approaches but none have translated into clinical practice. In 1970s, Glucose-insulin-potassium (GIK) solution was initially promoted for the treatment of acute myocardial infarction.[93] Following this, many clinical trials were performed, while this therapy showed promising results however, due to in-conclusive results further progression of this into clinical use was dampened.[93-95] Likewise a different group of compounds such as adrenergic receptor [98, 99, 100, 101] and calcium channel blocker[102, 103] were studied, which reduce workload on the heart and decreases the oxygen requirement. These drugs have been shown to only alleviate the symptoms and the risk of injury but couldn't reduce the mortality rate. Following this different pharmacological agents have been studied, like the Na^+/H^+, inhibitor cariporide. In intial clinical studies this drug was shown to be cardioprotective but still couldn't be translated into clinical use because, this drug was shown to cause neurological effects. [96, 97] Many other agents have been investigated which were reported to be protective in experimental animals; however, none have successfully translated into clinical practice with the exception of early reperfusion.[96, 109] Untill today, reperfusion stratergies have been used as a standard procedure to salvage the ischemic myocardium.[96, 109] Through this stratergy the moratility rate with acute myocardial infarction decreased, however it inconsistently and/or failed to limit or control the progression of infarction. The continuous failure in limiting IR injury raises the question of whether it will be possible to control or limit infarct size in man.

1.6.1 Ischemic Pre- and Post-conditioning

In 1986, Murry *et al* described the concept of ischemic preconditioning (IPC)[110], which entailed experimentally subjecting a canine heart to four 5 minute episodes of alternating coronary artery occlusion and reperfusion prior to a prolonged 40 min period of occlusion and a 4 day reperfusion period. Interestingly, there was a significant reduction in myocardial infarct size compared to the control group.[110] IPC is one of the most powerful and reproducible cardioprotective mechanisms identified to date. Although this method was demonstrated to be very effective, it is not practical clinically for therapeutic use because to render protection to a pre-existing myocardial patient intervention has to perform prior to the onset of the acute event.

In 2003, Zhao *et al* introduced a concept called ischemic postconditioning (IPost), as a cardioprotective strategy, which is similar to IPC but administered during reperfusion.[111] It was demonstrated that when canine hearts were subjected to three 30s episodes of coronary occlusion and reperfusion followed by 60 min of sustained occlusion, there was a significant reduction in myocardial infarction. Supporting this, clinical studies in patients undergoing percutaneous coronary intervention (PCI), where the angioplasty balloon is inflated and deflated have reported that IPost is very effective, similar to IPC, in rendering cardioprotection.[112-114] Though this method has been proven to be very effective it is stil not commonly practiced clinically because to render protection through this mechanism surgical intervention is needed.

1.7 Protective Pathways

Further in-depth mechanistic studies have revealed that both IPC and IPost render protection by numerous signal transduction pathways such as phosphatidylinositol 3-kinase (PI3K) [115] and ion channels like ATP sensitive potassium channels (K_{ATP}).[116] In addition to this ERK1/2, p38 MAPK and the JAK/STAT pathways have been shown to play a major role in conveying the protective signal however the contribution of these kinases is still controversial.[117]

1.7.1 Phosphatidylinositol 3 Kinase-Akt Pathway

Previously, Tong *et al* demonstrated that IPC elicited cardioprotective actions through a PI3K-Akt dependent pathway.[115] Subsequently, Hausenloy *et al* demonstrated a role for a PI3K-Akt pathway in IPost. [117, 118, 119] PI3K are lipid kinase enzymes that phosphorylate phospholipids at the 3' position. Phosphatidylinositols (PtdIns) are phospholipids that consist of a phosphoglyceride and inositol ring which can be phosphorylated and dephopshorylated by various lipid kinases or phosphataseenzymes.[120] PI3K phopshorylates these PtdIns resulting in generation of PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3.[120] PI3K exist in different classes and are classified based on their substrate specificity, mode of activation and molecular structure.[121-123] Class IA and class IB from class-I PI3Ks are an important group of enzymes and are ubiquitously expressed including the heart. These are heterodimeric enzymes composed of a regulatory adaptor subunit coupled to a catalytic subunit. [121-123] (Class IA is made up of p110α, p110β, and p110δ, whereas the class IB is made up of p110γ subunit). Though they exist in different classes they are known to

produce similar products, PtdIns phopshorylated at the 3rd position. PtdIns(3,4,5)P3 synthesized by class I PI3K, is an important precursor, which selectively activates many downstream effectors by binding to target domains such as the pleckstrin homology (PH) domain.[122] The levels of PtdIns (3,4,5)P_3 inside the cell are regulated by the lipid phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome 10). [122] Upon activation PTEN is recruited to the plasma membrane where it converts PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. [121-123] The negative regulation action of PTEN on PI3K signalling occurs in various regions in the body including the heart.[122] Recently it was demonstrated that PTEN gets activated following oxidative stress. [121-123]

Akt, a downstream target of PI3K, is a serine/threonine protein kinase that contains an N-terminal PH domain, a central catalytic domain, and a C-terminal regulatory domain.[122] It is activated in two steps, first it is initiated by recruitment of Akt to the membrane and binding to PtdIns(3,4,5)P3 by its PH domain and secondly, following this phosphorylation of a threonine residue at the 308 position in the catalytic site by PDK1 or a serine residue at 473 by a different enzyme which is still unknown.[120, 122, 123] Following activation it is known to control a variety of regulatory processes such as inhibition of apoptosis, induction of hypertrophy, promotion of cell proliferation and improvement of metabolism through activation/or inactivation of various downstream effectors such as GSK3β, p70S6K, endothelial NO synthase (eNOS) and Bcl-2 proteins (BAD).[115, 117-120, 122-126] Additional functional roles of PI3K include modulation of cardiac electrical activity through regulation of ion channels and exchangers.[122, 127-129 122,

[130, 131] However, whether this particular action is involved in rendering cardiac protection against IR injury is still not known.

1.7.2 Glycogen Synthase Kinase-3

GSK3 is a serine/threonine kinase that was originally known as an enzyme that phosphorylates and inhibits glycogen synthase. It exists in two isoforms, GSK3α and GSK3β; both isoforms are highly homologous and are found in the cytosol and nucleus. Under basal conditions GSK3 is highly active and is inactivated when phosphorylated, the phopshorylation sites are serine 21 for GSK3α and serine 9 for GSK3β.[122, 132, 133] Though these both isoforms are constitutively expressed, the role of GSK3α isoform is not known. On the other hand it has been reported that following IR injury active GSK3β increases the vulnerability of cells to ischemia reperfusion injury. Though the exact mechanism of action is not known it is thought that following IR injury it tranlocates to mitochondria and alters the mPTP function leading to collapse of mitochondrial membrane potential. Many studies support this hypothesis by using pharmacological inhibitors and knock out models to demonstrate that inhibition of GSK3β protects the cell from IR injury. IPC and IPost have also shown protection via inhibition of GSK3β. [122, 132-134]

1.7.3 ATP sensitive potassium channels

Plasma membrane ATP sensitive potassium channels

pmK_{ATP}, expressed at the plasma membrane, are made up of a pore forming subunit (Kir) and a channel regulating subunit, sulfonylurea receptors (SURs). They are composed of 8 protein subunits, 4 inwardly rectifying K+

subunits (Kir) forming the central pore and 4 sulfonylurea receptors (SUR). [116, 135] In the heart these channels are made up of the specific Kir6.2 and SUR2A isoform subunits.[116, 136, 137] These channels are closed by ATP under normoxic conditions and open under conditions of stress such as exercise or metabolic inhibition conditions such as hypoxia or ischemia. Opening of *pm*K_{ATP} channels is shown to shorten action potential duration, decrease Ca^{2+} overload inside the cell, and prevent mitochondrial damage and apoptosis following oxidative injury.[136-139] Using genetic altered mice which lack Kir6.2, the pore forming unit of the channel, and by use of pharmacological agents, it has been demonstrated that these channels are required for IPC and and that inhibition of these channels following IR injury abolishes the protective effect. [116, 140, 141]

Mitochondrial ATP sensitive potassium channels (mitoK_{ATP})

MitoK_{ATP} sensitive potassium channels are a different group of channels also assumed to be present on the mitochondria. Various studies have shown that these mitoK_{ATP} channels rather than the *pm*K_{ATP} channels are required to demonstrate the protective effect of IPC[142-145] by using the inhibitors such as 5-HD and activators such as pinacidil or diazoxide which are nonspecific inhibitors and activators of mitoK_{ATP}, respectively.[142, 143, 145-148] It has been demonstrated that activation of these channels causes an increase in mitochondria matrix volume, due to influx of K^+ ions followed by Cl^- ions through selective anion channels.[149, 150] Thus, rising matrix volume leading to close contact of mitochondrial membranes results in easy transport of ADP and activation of the respiratory chain and synthesis of ATP. In addition, activation of these channels

has been shown to decrease matrix Ca^{2+} overload by increasing K^+ ion influx and depolarising the mitochondrial membrane.[151] However the structure of the mitoK_{ATP} channel and the exact mechanism through which it renders protection is still not known.

1.7.4 Different ion channels present on the mitochondria

In addition to mitoK_{ATP} channels, other types of potassium channels are present on the membrane of the mitochondria, which have also been demonstrated to be involved in rendering protection against oxidative stress, ion channels such as calcium activated K^+ channels (BK_{Ca}^{2+}) and Kv1.3 voltage gated potassium channels. [147] Studies have demonstrated that these ion channels are activated when the mitochondrial membrane potential is depolarised and render protection by decreasing mitochondrial Ca^{2+} overload and improve energy production following stress induced injury, however the mechanism of action is still not known.[152, 153]

IPC and IPost are the most powerful cardioprotective mechanisms identified to render protection against IR injury. So far these mechanisms are consistent in limiting infarct size as demonstrated in different animal models. The discovery of IPC and IPost, the two most important forms of cardiprotective mechanisms have broadened our knowledge of signalling mechanisms that can salvage the myocardium following IR injury and have encouraged investigators to scientists a new alternative approach to protecting the myocardium following injury. This led to identification of various classes of receptors that can mediate these protective pathways such as adenosine, opioid and bradykinnin receptors.[96,

[109, 141, 154-158] Pharmacological agents have been identified that can mediate these pathways either directly or through activation of these receptor. These include insulin, ATP-sensitive potassium channel openers, volatile anesthetics, nitric oxide.[96, 109, 116, 140, 141, 157] All of these agents, when used in preclinical animal models, offered significant protection to cardiac function and structure following IR injury and offer benefits over the drawbacks of IPC, IPost and intermittent cross-clamping. In spite of having advantages over IPC and IPost many of these agents couldn't be translated to clinical therapy first because of inconsistent results at the preclinical level, second due to lack of proper information on the mechanism of action and third uncertainty over the safety of the drug. [96, 109] Despite intense research efforts, there remain few if any effective therapies which ahve been successful in initial clinical trials. Thus, novel therapies aimed at reducing myocardial damage and improving clinical outcomes following IR injury are still required.

1.8 Thesis Overview

1.8.1 Rationale

Over the past few years, various studies have demonstrated that metabolites of AA acids, EETs act as critical intracellular mediators, decrease apoptotic cell death, improve postischemic left ventricular functional recovery and reduce myocardial infarct size following IR injury. However, the exact mechanism of action through which EETs render protection is still not clear. Previous work from our laboratory has demonstrated that EETs render protection by activating the PI3K pathway. In addition, others have shown that *pm*K_{ATP} channels are also involved in this process, however the role of each of the above components in the EET mediated protective mechanism is not known.

During cardiovascular ischemia reperfusion injury, damage of key organelles like the mitochondria is known to be a important contributor for the development of pathogenesis. Maintaining mitochondrial integrity is important for homeostasis of the cellular environment and also normal organ function. The overall aim of the current study is to investigate whether EETs render cardioprotection by limiting mitochondrial damage and function following IR injury and to identify the role of PI3K and *pm*K_{ATP} channels in this protective mechanism.

1.8.2 Hypothesis

The global hypothesis for this thesis is:

Increased amounts of EETs following ischemic reperfusion injury will provide cardioprotective responses, which prevent mitochondrial damage and maintain cardiac function.

The specific hypotheses are as follows:

- Altering the amount of EETs using pharmacological agents, such as soluble epoxide hydrolase inhibitors or EET analogs, trigger cardioprotective signals and improve functional recovery against IR injury
- EETs attenuate EKG abnormalities and improve ventricular repolarization following IR injury through activation of K_{ATP} channel.
- EET-mediated cardioprotection against IR injury reduces mitochondrial damage through PI3K dependent *pm*K_{ATP} channel activation mechanism.

1.8.3 Thesis Aim

1. To determine if a pharmacological inhibitor of sEH, is cardioprotective against ischemia/reperfusion injury
2. To investigate the role of *pm*K_{ATP} in EET-mediated improvement in postischemic ventricular function
3. To investigate the cardioprotective effect of a dual acting EET analog and role of PI3K in EET-mediated cardioprotection
4. To determine the role of PI3Kα and *pm*K_{ATP} in the mechanism of EET mediated cardioprotection

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CHAPTER-II

Epoxyeicosatrienoic Acids Regulate Mitochondrial Function Following Stress Through Activation of Plasmamembrane K_{ATP} channels[2]

[2] *A version of this chapter will be submitted to Canadian Journal of Physiology and Pharmacology: Batchu SN, Zhang.Y, Lee SB, Cho WooJung, El-Sikhry H, Weldon S, Seubert JM. Epoxyeicosatrienoic Acids Regulate Mitochondrial Function Following Stress Through Activation of Plasmamembrane K_{ATP} channels.*

2.1 Introduction

Plasmamembrane ATP-sensitive K^+ channels (pmK_{ATP}) belong to group of inward rectifiers that sense the metabolic condition of the cell and act to protect the cell.[1] Activation of pmK_{ATP} channels are known to shorten the repolarisation phase of the action potential, hyperpolarize the cell and limit calcium entry.[2-4] Several studies using Kir6.2 channel deficient mice or pmK_{ATP} inhibitors have demonstrated that these channels are important for ischemic preconditioning [5, 6] and produce beneficial effects on the myocardium following ischemic injury.

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acid (AA).[7-9] They are potent intracellular lipid mediators and exist as four regioisomeric metabolites, 5, 6- , 8, 9- 11, 12- and 14, 15-EETs.[10, 11] Levels of these metabolites are modulated by different mechanisms: by reincorporating themselves into phospholipid membranes or by β-oxidation to smaller reactive epoxides. However, the predominant pathway is metabolism to the less active vicinal diol compounds, dihydroxy epoxyeicosatrienoic acids (DHETs), by soluble epoxide hydrolase (sEH). [7, 12, 13] In the mouse model, targeted disruption of the epoxide hydrolase gene has been shown to decrease the hydrolysis of EETs and render protection against ischemia-reperfusion (IR) injury.[14-16] Similar cardioprotection was seen when hearts were treated with pharmacological sEH inhibitors (sEHi). [15, 17]

We have demonstrated previously that elevated levels of EETs render protection through activation of pmK_{ATP} [18-20], by regulating mitochondrial function and by delaying the opening of the mitochondrial permeable transition

pore (mPTP).[21] Moreover, it was demonstrated recently that *pm*K_{ATP} channels are required for EETs to maintain mitochondrial function; however, the exact mechanism is still not known. [22]

In the present chapter, we examined the protective actions of three structurally divergent novel sEHi's BIX01960, BIX01961 and BIX01962. Following those intial studies we conducted animal and cellular studies to identify the interaction between the *pm*K_{ATP} channels and mitochondria. Data from the chapter demonstrate that elevation of EETs concentration render protection against IR injury by regulating mitochondria function following stress, by preventing or slowing the opening of mPTP via activation of *pm*K_{ATP} channels.

2.2 Materials and methods

2.2.1 Animals

Commercially available B6129SF2 and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice with targeted deletion of *Ephx2* gene coding for soluble epoxide hydrolase (KO) were obtained from Boehringer Ingelheim Pharmaceuticals, Inc, USA. The heterozygous *EPHX2* founder mice were generated for Boehringer Ingelheim Pharmaceuticals Inc. at Lexicon Genetics Inc. by gene trapping using random insertional mutagenesis with retroviral vector VICTR48 as described by Zambrowicz *et al*. [23] The integration site of the targeting cassette is in intron two. The heterozygous *Ephx2* mice were then mated to establish a colony in the C57BL/6 albino × 129Sv/Ev mixed genetic background. Once obtained, *Ephx2* null homozygotes were paired for subsequent breeding. All studies were carried out using mice, aged 3-4 months, weighing 25-35g. Experiments were conducted according to strict guidelines provided by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS).

2.2.2 Isolated heart perfusion

Mouse hearts were perfused in the Langendorff mode as described. [16, 18, 24] Briefly, the hearts were perfused in a retrograde fashion at constant pressure (90cmH_2O) with continuously aerated (95%O_2/5%CO_2) Krebs-Henseleit buffer at 37^oC. Hearts were perfused for 40 min (stabilization), and then subjected to 20 or 30 min global no-flow ischemia, followed by 40 min reperfusion. In some experiments, hearts were first stabilized for 20 min, then perfused with either of the sEH enzyme inhibitors BIX01960, BIX01961, BIX01962 (0.1-10μM, Boehringer Ingelheim

Pharmaceuticals, Inc, USA), 11,12-EET (1μM, Cayman Chemicals, USA) or vehicle for 20 min, then subjected to ischemia and reperfusion. In other experiments, the non selective *pm*K_{ATP} inhibitor, glibenclamide (GLIB, 10μM), or the mPTP opener, atracyloside (Atr, 50μM), were administered during the 40 min reperfusion. Recovery of contractile function was measured as left ventricular developed pressure (LVDP) at 40 min reperfusion expressed as a percentage of preischemic LVDP. To determine the amount of infarction, hearts were reperfused for 2 h, incubated with a 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 and comparing with the whole slice area.

2.2.3 Cell Culture Experiments

H9c2 cells (American Type Culture Collections, Manassas, VA) were cultured in 75cm^2 flasks at 37°C in an atmosphere of 5% CO_2/ 95% air. Dulbecco's modified Eagle medium (DMEM) with phenol red, supplemented with 10% bovine serum albumin and antibiotics such as penicillin, streptomycin and amikacin, were used to nourish the cells. In all the experiments, cells were treated with 11, 12-EET (1μM), BIX01962 (1μM) or GLIB (10μM) in DMEM. Anoxic conditions, 37°C in an atmosphere of 5% CO_2/ 95% N_2, were generated in an air tight chamber (MIC101, Billups-Rothenberg, Inc.). These conditions were maintained for 6 h followed by 30 min or 16 h reoxygenation. Cell viability was assessed by using a 0.4% trypan blue dye exclusion assay.

2.2.4 Caspase assay

Caspase-3 activity was assessed in cytosolic fractions from the H9c2 cells by monitoring the release of 7-amino-4-methylcoumarin (AMC) by proteolytic cleavage of the peptide Ac-DEVD-AMC (20μM) (Sigma-Aldrich, Oakville, ON) as previously described.[25] Fluorescence was monitored at wavelengths of 380 nm (excitation) and 460 nm (emission). Specific activities were determined to be within the linear range of a standard curve established with AMC.

2.2.5 Immunoblotting

Subcellular fractions were prepared from frozen mouse hearts and cells as previously described.[15, 26] Protein quantities were determined using a Bradford protein assay kit (BioRad Laboratories, Canada), were resolved on 12%SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies to phosphorylated and/or total ERK1/2 (1:1000), t-BAD (1:500) (Cell Signalling Technology, Inc. USA) and prohibitin (1:1000) (Fitzgerald, Concord, MA, USA). Relative band intensities were expressed in arbitrary units assessed using Image J software (USA, NIH, Bethesda, MD).

2.2.6 Reactive Oxygen Species

Reactive oxygen species (ROS) were measured by incubating the H9c2 cells with 2.5μM CM-H_2DCFDA (5-(and-6)-chloromethyl– 2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester) (Invitrogen, USA) in Hank's buffer following anoxia-reoxygenation. Changes in fluorescence were recorded in H9c2 cells treated with vehicle, 11, 12-EET (1μM), BIX01962 (1μM) or H_2O_2

(100μM) using a fluorescence plate reader (excitation 485±20nm, emission 590±30nm).

2.2.7 Mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was determined by loading H9c2 cells with 150 nM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen, USA). TMRE is a cationic dye attracted to polarized mitochondria membranes, a reflection of mitochondrial function. Cells were incubated with 150 nM TMRE for 20 min after 6 hr anoxia and 16 hr reoxygenation. Changes in fluorescence were recorded at 37ºC using a Zeiss Axio Observer Z1 inverted epifluorescence microscope with a 500ms exposure time. Cells were observed under a PlanApo 40x oil immersion objective lens (Zeiss) with a numerical aperture value of 1.4. TMRE was excited at 555 nm and emission was recorded with a band-pass filter of 575–640 nm. Measurements were taken from individual experiments and intensities were quantified relative to background.

2.2.8 Statistical analysis

Values are expressed as the mean ± SEM. Statistical significance between the groups was determined by use of the Student's *t* test and one way ANOVA followed by Duncan's test to assess multiple group comparisons. Values were considered significant if $P < 0.05$.

2.3 Results

2.3.1 Cardiac Performance after Ischemia-Reperfusion in Perfused Hearts

To determine whether decreasing the hydrolysis of EETs to DHETs through pharmacological inhibition of the sEH enzyme protects the heart from ischemia reperfusion injury, hearts from B6129SF2 mice were perfused with three different sEH inhibitors: BIX01960, BIX01961 and BIX01962. Inhibitors were perfused for 20 min before ischemia and throughout the 40 min reperfusion period (Fig. 2-1A) at three different concentrations (0.1, 1 and 10μM) (Fig.2- 2A). All the hearts had normal baseline contractile function, measured either as LVDP or rate-pressure product (RPP=LVDPxHR) (Table.1-1). Hearts perfused with BIX01960 (0.1 and 1μM) showed an improved postischemic recovery of LVDP (BIX01960 0.1 μM: 60.0±3.3%; BIX01960 1μM: 68.3±4.3%) compared to vehicle treated hearts (42.0±4.3%). Perfusion with the highest concentration of BIX01960 (10μM) (26.3±4.3%) did not improve the recovery of LVDP. BIX01961 did not show any significant improvement in LVDP at all three concentrations (0.1μM, 1μM and 10μM) compared to vehicle treated animals. On the other hand, BIX01962 demonstrated a dose-dependent improvement of postischemic LVDP (Fig. 2-2A) with the maximal recovery occurring at the highest concentration (1μM 63.0±8.3%; 10μM 68±7.3%). This recovery is similar to the data obtained from WT hearts perfused with 11,12-EET(1μM) or hearts from sEH KO animals (Fig. 2-3A), thus suggesting that the sEHi, BIX1962, is a more potent agent compared to the other inhibitors.

In contrast, the IC_{50} and K_i values of BIX01960 and BIX01961 compounds indicate that these 2 agents are more potent than BIX01962 in inhibiting sEH (Table2-2). Looking at the functional recovery of BIX01960 at the highest concentration and BIX01961 at all three concentrations showed an inhibitory effect; this may be due to other offsite actions of the drugs apart from their sEH inhibitory properties.

To further assess the cardioprotective effects of BIX01962, infarct size was assessed after 20 min of global ischemia and 2 h of reperfusion. Infarct size was measured as a percentage of the infarcted region to the area at risk (IS/AAR). A significant decrease in infarct size was observed in the hearts treated with BIX01962 compared to vehicle-treated hearts (Fig. 2-3B).

2.3.2 Role of the mitochondrial permeable transition pore (mPTP)

Following IR injury, proper functioning of mitochondria is important for the restoration of cardiac function. mPTP is one of the important determinants that dictates mitochondrial function following injury.[27] To determine whether EET or the elevation of EETs by sEHi mimics mediates cardioprotection by regulating opening of mPTP, we perfused hearts from C57BL6 mice with 11, 12-EET (1μM) or BIX01962 (1μM) in the presence or absence of the mPTP opener, Atr. Perfusion of BIX01962 (1μM) these hearts to improved the recovery of LVDP similar to that of C57BL6 hearts perfused with 11, 12-EET (1μM) (Cont: 34.0±3.0%; Cont+11, 12-EET: 70±9.0%; Cont+BIX01960: 55.0±9.0%). But when Atr (50μM) was co-perfused through the hearts, this improved recovery was

abolished (Cont+11, 12-EET+Atr: 34±4.0%; Cont+BIX01960+Atr: 24.0±7.0) Atr did not affect the functional recovery of the WT control heart (Fig.2- 4A).

2.3.3 Role of pmK_{ATP} channels in functional recovery

Ca^{2+} is the one of the key factors that causes damage to heart against IR injury. Activation of *pm*K_{ATP} is known to provide protection against stress by regulating Ca^{2+} overload. [28, 29] To verify whether BIX01962 protects through this mechanism, WT hearts perfused with BIX01962 (1µM) were co-treated with GLIB (10µM). In this case, GLIB completely abolished the BIX01962 improved LVDP (Fig.2-5A).

2.3.4 Pro-apoptotic protein BAD expression

BAD, a pro-apoptotic protein belongs to the Bcl-2 family. Following IR injury, when there is an increase in intracellular Ca^{2+} overload, it is known to become activated and move to mitochondria.[30] To investigate the role of BAD in the cardio-protective mechanism, we looked at the expression of BAD protein in the mitochondrial fractions of the perfused hearts. Following IR injury, a greater expression of BAD was observed in the mitochondrial fractions separated from the WT control hearts. Upon perfusing the hearts with BIX01962 (1µM), the expression of BAD in the mitochondria fraction was decreased (Fig. 2-5B).

2.3.5 Anoxia-Reoxygenation

To further investigate the molecular mechanism(s), cellular studies were performed in H9c2 cells looking at stress markers like p-ERK1/2, ROS and mitochondrial $\Delta\psi_m$, which are altered when there is change in the intracellular Ca^{2+} levels or when the mPTP is opened . When cells were subjected to 6 h of

anoxia followed by 30 min reoxygenation, there was no difference in the percent of cell viability between the control and treated groups (data not shown). However, the expression of stress activated kinase p-ERK1/2 and generation of ROS was significantly higher in the control group compared to treated groups (Fig. 2-6A & 7A). Similarly, when cells were subjected to 6 h anoxia followed by 16 h of reoxygenation, generation of ROS and caspase-3 activity were still significantly high and the percentage of viable cells was significantly less in the control group compared to the treated groups (Fig. 2-7B, 8A & 8B). However, the expression of p-ERK1/2 was less in the control group compared to the treated group (Fig. 2-6B).

Consistent with the above data, loss of mitochondrial function, represented by dissipation of $\Delta\Psi m$, was very rapid in the control group. When cells were treated with 11, 12-EET (1μM) or BIX01962 (1μM) the dissipation of $\Delta\Psi m$, was slowed. Interestingly, when cells were co-treated with GLIB (10μM) this effect was abolished (Fig. 2-9A). Therefore, these results with the functional data suggest that BIX01962 propably render protection by activating *pm*K_{ATP} channels and preventing Ca^{2+} mediated opening of mPTP mechanism.

2.4 Discussion

In this chapter, we present data demonstrating the cardioprotective properties of the pharmacological sEH inhibitor, BIX01962; its role in improved cardiac functional recovery and reduced infarct size following ischemic injury. In addition, our chapter demonstrates that EETs produce cardioprotection through the activation of *pm*K_{ATP} channels via regulation of mitochondria function.

sEH is known to catalyze the hydrolysis of a wide range of mutagenic, carcinogenic and toxic epoxides into diols. [31, 32] Endogenously derived fatty acid epoxides like EETs are also known to be hydrolyzed by sEH. [32] In-vivo and ex-vivo studies using genetic modified mice produced by targeted deletion of the *Ephx2* gene coding for sEH was reported to produce anti-hypertensive and anti-arrhythmic effect, and render protection against IR injury. [16, 33] Studies with pharmacological sEHi have also shown a similar trend in the improvement of cardiac parameters.[15, 17] This cardioprotective effect has been attributed to decreased hydrolysis of EETs to DHETs. In the present chapter, looking at the cardioprotective effects following IR injury, we characterized three pharmacological sEHi's (BIX01960, BIX01961 and BIX01962) that are similar in potency (IC_{50}) to urea based established inhibitors like TUPS, AUDA and AUDA-BE.[34] Upon perfusing the hearts with BIX01962 (IC_{50}= 15nM), we noticed improved postischemic left ventricular functional recovery and decreased infarct size. BIX01960 (IC_{50}= 6.6nM) showed a similar protective effect to that of BIX01962 at lower concentrations, but at the higher concentration (10µM), it didn't improve functional recovery. Interestingly, in spite of being a very potent

sEHi, BIX01961 (IC_{50}=6.4nM) didn't improve postischemic functional recovery. Though the reason for the lack of effect is not clear, we assume that this may be due to some off site actions of the drug. Data obtained from the BIX01962 suggest that although it is less potent compared to the other two BIX sEHi, but following IR injury it improved postischemic functional recovery and reduced infarct size similar to that of other optimized pharmacological inhibitors and genetic modified models.

Damage to mitochondria following IR injury is known to be one of the important factors responsible for causing pathogenesis. [29] Studies have shown that reperfusion following ischemia stimulates the opening of mPTP. [28, 29] It has been illustrated that this transition in the function of the mPTP following IR or oxidative stress allows the passage of any molecule <1.5kDa into the matrix of mitochondria, leading to the uncoupling of oxidative phosphorylation, generation of ROS, release of cytochrome C and cell death, which ultimately results in decreased cardiac function.[29] Previously, we have shown that following oxidative stress EETs have been shown to render protection by slowing or preventing the opening of mPTP. [21] In the present study, we have seen that, following IR injury, hearts perfused with EETs or elevation of EETs by preventing its hydrolysis by inhibiting sEH with BIX01962 showed improved functional recovery and decreased infarct size compared to control hearts. Interestingly, when these hearts were co-perfused with an mPTP opener, Atr (50μM), the improved recovery was abolished. These data further support our previous findings that EETs produce

protection by preserving mitochondrial function, which occurs by preventing the opening of the mPTP.

Following ischemia and reperfusion, a raise in intracellular Ca^{2+} is known to be the one of the key factors responsible for opening of the mPTP. [28, 29] Though the exact mechanism is not known, in vitro studies have demonstrated that the elevated cytosolic Ca^{2+} enters mitochondria through a uniporter and accumulates in the matrix. [28, 29] This excess Ca^{2+} is known to enhance the opening of the pore by degrading or reducing the adenine nucleotide binding to adenine nucleotide translocase, or by activating cyclophilin D binding to adenine nucleotide translocase, one of the components of the mPTP leading to opening of the transition pore.[29] Other studies have demonstrated that the elevated intracellular Ca^{2+} activates the pro-apoptotic protein BAD, which can initiate the opening of mPTP by translocating from the cytosol to mitochondria. [35] Marinovic *et al* have recently demonstrated that following oxidative stress, activation of *pm*K_{ATP} channels prevents intracellular Ca^{2+} overload and mitochondrial damage, signifying a unique protective role by these channels. [36] *pm*K_{ATP} channels are membrane stabilizers, and studies have shown that activation of these channels regulates the intracellular Ca^{2+} levels [2, 3, 5, 6] EETs are potent activators of *pm*K_{ATP} channels. We and others have shown previously that they decrease intracellular Ca^{2+} overload and render cardioprotection by activating these channels. [18-20] In this current chapter we have seen that following oxidative stress cells treated with EETs or BIX01962 has maintained $\Delta\Psi_m$, and decreased expression of stress activated kinase p-ERK1/2 (Ca^{2+} overload), ROS generation, caspase-3 activity

and cell death compared to controls. Additionally, hearts perfused with BIX01962 have decreased expression of BAD in mitochondrial fractions, suggesting that EETs or BIX01962 render protection by decreasing the intracellular Ca^{2+} load following stress. Interestingly, following oxidative stress, the maintained mitochondrial membrane potential seen in the EET or BIX01962-treated groups was abolished when co-treated with GLIB similar to our previous studies. Together with the above functional, cellular data and results from our previous studies, this suggests that EETs regulate mitochondrial function via regulating Ca^{2+} levels and inhibiting opening of mPTP through activation of *pm*K_{ATP} channel.

In summary, the data from this chapter demonstrates that BIX01962 improves functional recovery and reduces infarct size following IR injury, attributes of EETs. Moreover the data also demonstrate that elevation of EETs provide this protection by maintaining mitochondrial function by regulating the opening of mPTP by preventing Ca2+ overload at least partially through activation of *pm*K_{ATP} channels. Although the present chapter demonstrates a vital protective mechanism of EETs in protection against ischemic injury, further studies are required to elucidate additional mechanisms.

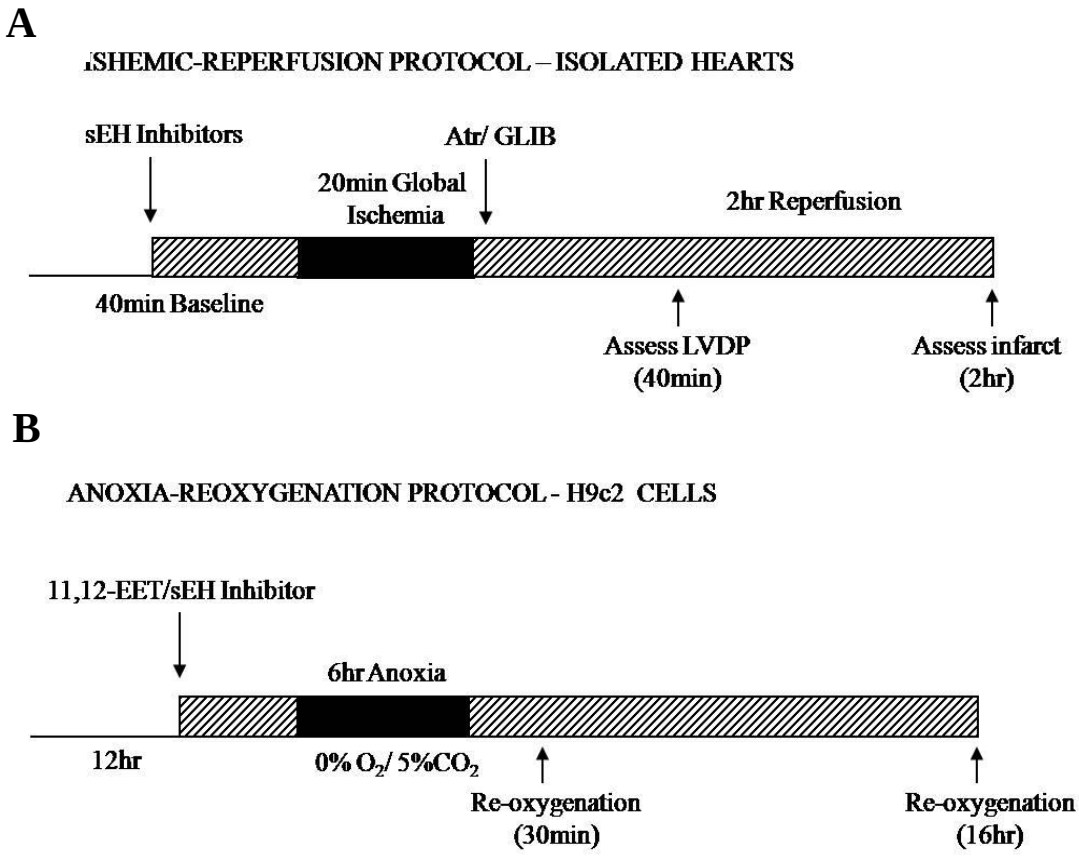

Fig. 2.1 (A), Schematic of the experimental protocol with the time course of drugs perfused to investigate LVDP and infarct size following ischemia and reperfusion. **(B)**, Schematic of the experimental protocol for anoxia reoxygenation experiments.

A

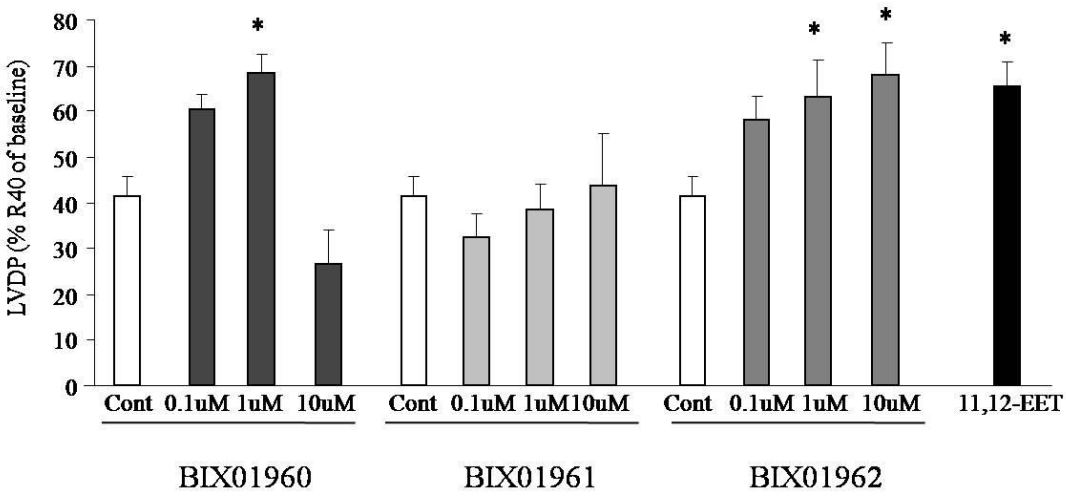

Fig. 2.2 (A), Histogram of the percent recovery of LVDP at 40 min reperfusion compared to baseline from the hearts perfused with vehicle or BIX01960, BIX01961, BIX01962 at increasing concentrations (0.1, 1 and 10μM) before ischemia and during reperfusion. Values represent mean±SEM, n=3-17 per group; *, $P<0.05$ vs. vehicle control.

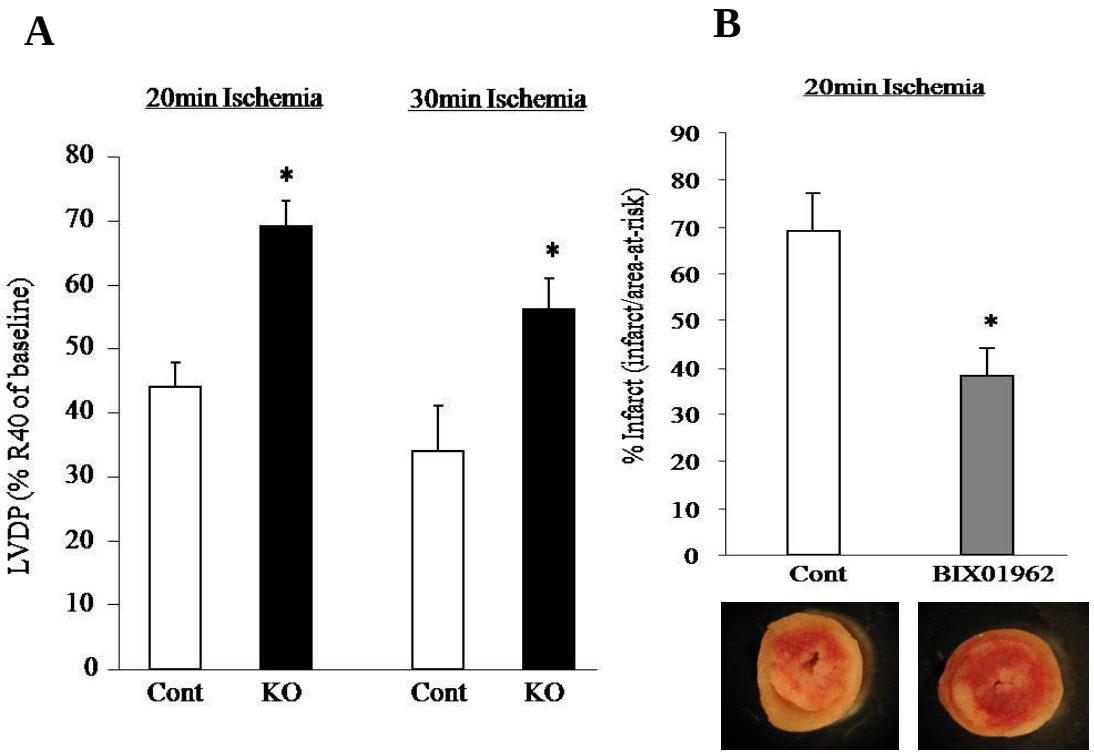

Fig 2.3 (A), Histogram of the functional recovery at 40 min reperfusion expressed as percentage of baseline LVDP from sEH null (KO) and WT (Cont) hearts following 20 min or 30 min ischemia. Values represent mean±SEM, *n*= 6-9 per group; *, *P*<0.05 vs. vehicle control. **(B)**, Quantification of infarct size from WT hearts perfused with vehicle or BIX01962 (1µM) following 20 min ischemia. Values represent mean±SEM, *n*=4-5 per group; *, *P*<0.05 vs. vehicle control of same genotype.

A

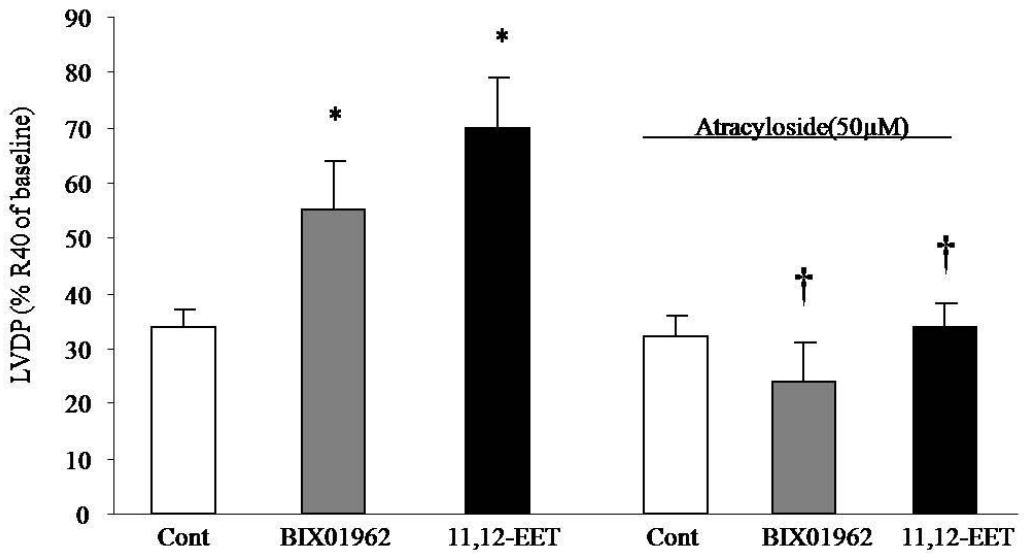

Fig. 2.4 (A), Histogram of the percent of LVDP change at 40 min reperfusion compared to baseline from hearts perfused with vehicle, BIX01962 (1µM) or 11,12-EET (1µM), with or without Atr (50µM). Values represent mean±SEM, *n*= 4-5 per group; *, *P*<0.05 vs. vehicle control. †, *P*<0.05 vs.BIX01962 treated group.

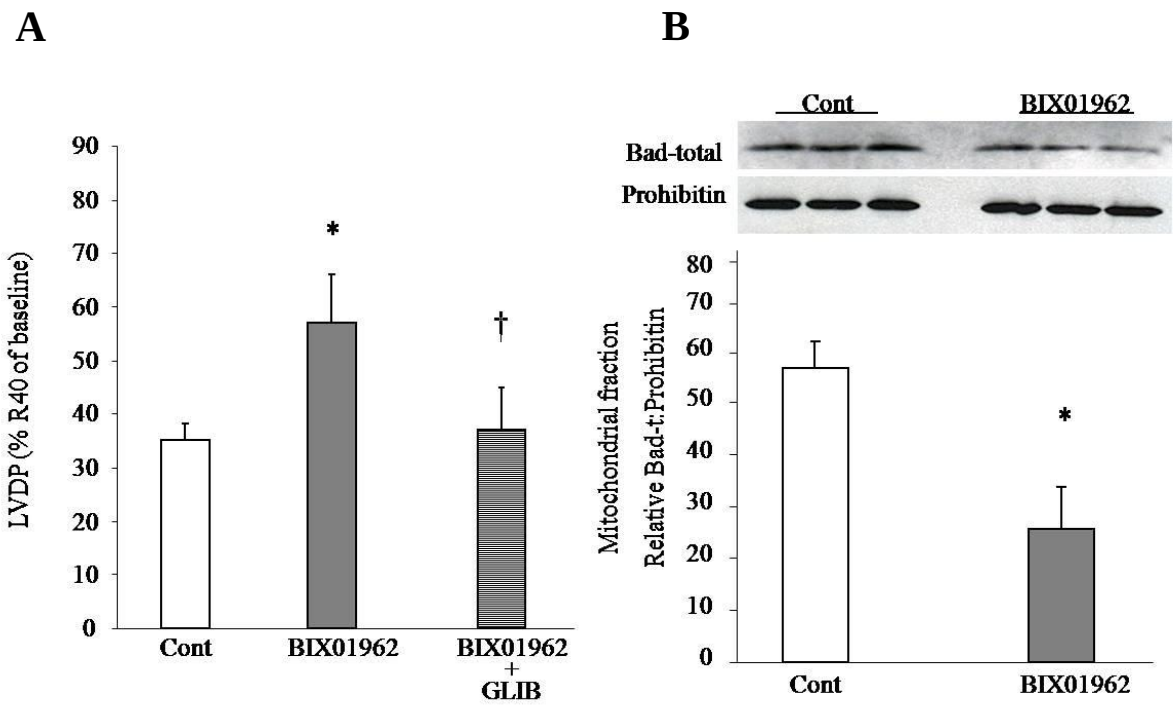

Fig. 2.5 (A), Histogram of the percentage of LVDP change at 40 min reperfusion compared to baseline from hearts perfused with vehicle, BIX01962 (1µM) or GLIB (20µM). Values represent mean±SEM, n= 4-5 per group; *, P<0.05 vs. vehicle control. †, P<0.05 vs. BIX01962 treated group. **(B)**, Immunoblot and densitometry showing the ratio of t-BAD to prohibitin expression from hearts subjected to 20 min of ischemia followed by 40 min reperfusion and treated with vehicle or BIX01962 (1µM)**.** Values represent mean±SEM, n=3 per group; *, P<0.05 vs. vehicle control.

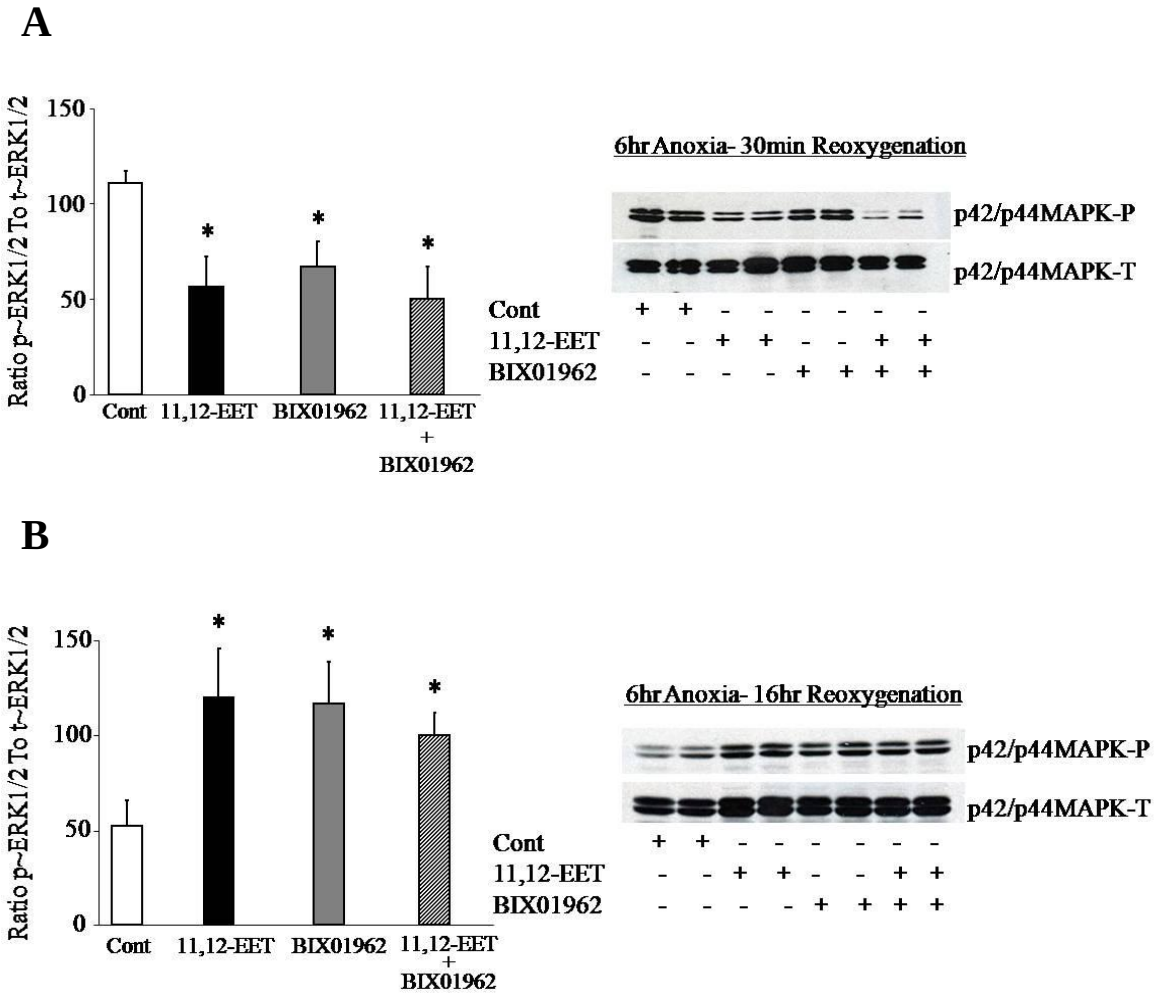

Fig. 2.6 (A). Immunoblot and densitometry showing the ratio of phospho-ERK1/2 to total- ERK1/2 expression from H9c2 cells treated with vehicle, 11,12-EET (1µM) or BIX01962 (1µM) following 6 h of anoxia and 30 min reoxygenation**.** Values represent mean±SEM, n=4-6 per group; *, P<0.05 vs. vehicle control. **(B)**, Immunoblot and densitometry showing the ratio of phospho-ERK1/2 to total-ERK1/2 expression from H9c2 cells treated with vehicle, 11,12-EET (1µM) or BIX01962 (1µM) following 6 h of anoxia and 16 h reoxygenation**.** Values represent mean±SEM, n=3-4per group; *, P<0.05 vs. vehicle control.

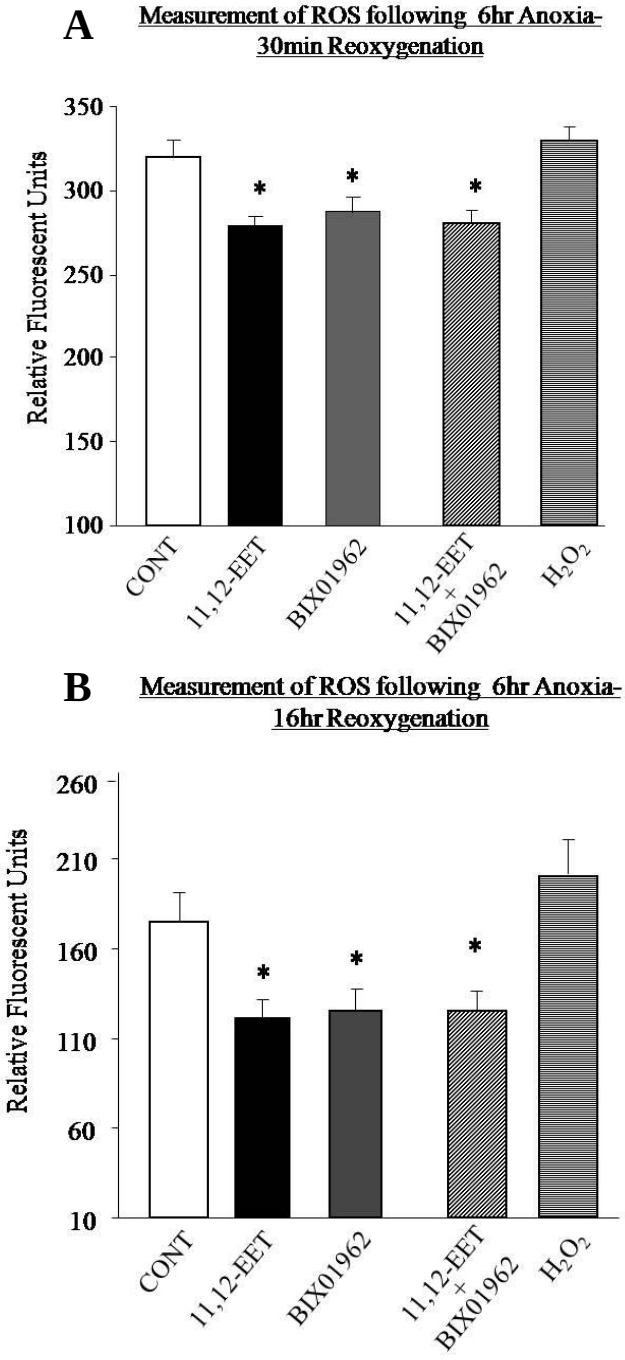

Fig 2.7 (A), Levels of ROS generated inside H9c2cells following 6hr of anoxia and 30 min reoxygenation. Values represent mean±SEM, *n*=3per group; *, *P*<0.05 vs. vehicle control. **(B)**, Levels of ROS generated inside H9c2cells following 6 h of anoxia and 16 h reoxygenation. Values represent mean±SEM, *n*=3per group; *, *P*<0.05 vs. vehicle control.

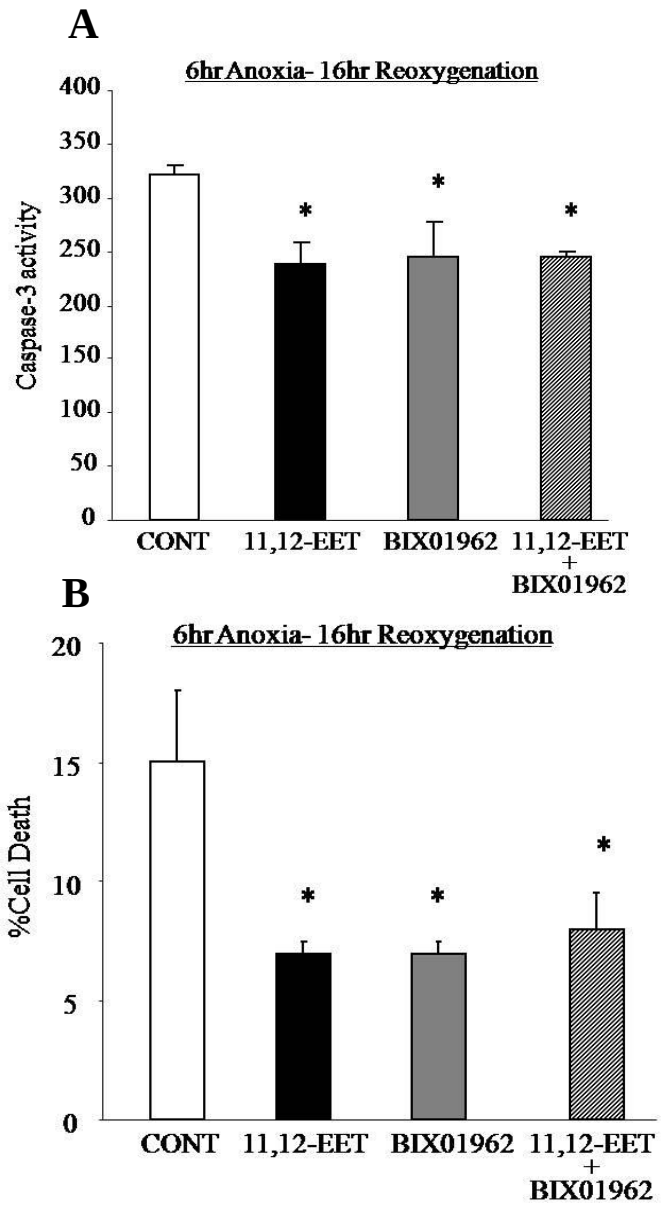

Fig. 2.8 (A), Histogram representing the caspase-3 activity from cells treated with vehicle, 11,12-EETs (1µM) or BIX01962 (1µM) following 6 h of anoxia and 16 h reoxygenation**.** Values represent mean±SEM, n=3 per group *, P<0.05 vs. vehicle control. **(B)**, Histogram representing the percentage of cell death from cells treated with vehicle, 11, 12-EETs (1µM) or BIX01962 (1µM) following 6 h of anoxia and 16 h reoxygenation**.** Values represent mean±SEM, n=4-6 per group *, P<0.05 vs. vehicle control.

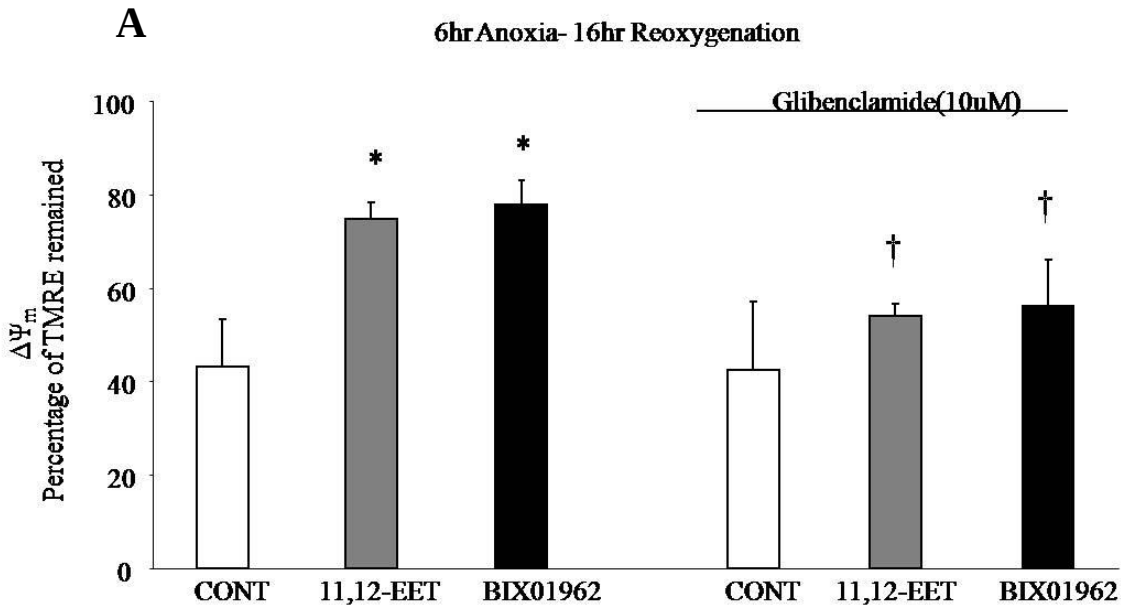

Fig. 2.9 (A), Histogram representing the percentage of membrane potential remaining (TMRE) from cells treated with vehicle, 11, 12-EETs (1µM) or BIX01962 (1µM), with or without GLIB (10µM) following 6 h of anoxia and 16h reoxygenation**.** Values represent mean±SEM, n=4-6 per group *, P<0.05 vs. Vehiclecontrol.

	Cont(n=18)	BIX01960(n=4-5)			BIX01961(n=4-5)			BIX01962(n=5-7)		
Isolated Perfused Heart Preischemic Baseline	0μM	0.1μM	1μM	10μM	0.1μM	1μM	10μM	0.1μM	1μM	10μM
LVDP (cmH_2O)	101±5	69±11*	91±7	71±11*	111±17	83±9	81±7	111±14	99±3	106±7
Rate of contraction dP/dt_{max} (cmH_2O/msec)	3901±254	2545±584	3643±450	2650±472	5030±603	4505±695	4167±341	4612±729	3923±536	3921±486
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec)	-3401±232	-1906±422*	-3070±267	-2251±441	-4368±748	-4347±529	-3392±408	-3737±571	-3558±409	-3485±331
HR, perfused (beats/min)	347±33	311±40	388±25	324±32	380±4	370±9	364±17	278±30	377±21	372± 20
Isolated Perfused Heart Postischemic Baseline										
LVDP (cmH_2O)	42±6	43± 8	61± 4	17± 5	38±12	33± 8	37±11	67±12	65±9*	71±9*
Rate of contraction dP/dt_{max} (cmH_2O/msec)	1415±194	1602±307	2388±393	577±150	1265±320	1037±134	1207±366	2057±414	1995±412	2091±197
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O/msec)	-1369±184	-1360±288	-1934±208	-480±132	-1311±460	-1089±174	-1042±312	-1817±365	-2036±323*	-2045±189*
HR, perfused (beats/min)	349±25	320±16	355±17	291±30	358±6	317±22	314±21	275±27	329±26	336±17

Table 2.1 Hemodynamic parameters measured in isolated perfused hearts following perfusion with sEH inhibitors at different doses. Values represent mean ± SEM,* P<0.05 vs Vehicle control

Compound	Molecular Activity [nM]			
	h-sEH		r-sEH	
	IC_{50}	K_i	IC_{50}	K_i
BIX01960	6.6	0.53	7.4	0.09
BIX01961	6.4	0.60	5.3	0.07
BIX01962	15.0	2.94	16.0	2.47

2.2 IC50 values of different sEH inhibitors.

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CHAPTER-III

Epoxyeicosatrienoic Acid Prevents Postischemic Electrocardiogram Abnormalities in an Isolated Heart Model[3]

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3.1 Introduction

Heart disease and stroke are major causes of illness, disability and death in Western societies. [1] As populations' age and co-morbidities such as obesity and diabetes become more prevalent, increased costs to heath care systems can be anticipated. Ischemic heart disease (IHD), also known as coronary artery disease (CAD) or coronary heart disease (CHD), results from the damage incurred from the reductions in blood flow and oxygen supply to the heart. Resultant alterations in ionic homeostasis in the ischemic myocardium alters resting membrane potential, the action potential duration (APD) and myocyte excitability resulting in EKG abnormalities. [2-5] Classical electrocardiographic (EKG) abnormalities associated with ischemic injury are prolonged QT interval and ST-segment elevation (STE) [6, 7], where QT prolongation reflects a decreased repolarization following depolarization of the cardiac ventricles, whereas STE provides a measure of ischemic injury. These EKG changes can manifest as serious arrhythmias, thereby impeding cardiac function, with possible fatal outcomes.

Arachidonic acid (AA), an essential polyunsaturated fatty acid found esterified to membrane phospholipids, may be released by phospholipases following stress stimuli such as ischemia. [8] Free AA can then be metabolized by CYP epoxygenase to four regioisomeric eicosanoid metabolites, epoxyeicosatrienoic acids (5, 6-, 8, 9-, 11, 12-, and 14, 15-EET). [9, 10] Conversion to the corresponding dihydroxyeicosatrienoic acids (5, 6-, 8, 9-, 11, 12-, and 14, 15-DHET) by soluble epoxide hydrolase (sEH) reduces their biological activity. EETs are important components of many intracellular signalling pathways in both

cardiac and extra cardiac tissues. They activate various stress response systems, such as p42/p44 mitogen activated protein kinases (MAPK); enhance membrane ion channel activity, such as K^+ channels; and improve postischemic recovery of left ventricular function. [11-14]

Activation of plasmamembrane K_{ATP} channels (*pm*K_{ATP}) can protect the heart against ischemic reperfusion injury by hyperpolarizing the cell, thereby limiting calcium entry, preserving ATP utilization, and maintaining cardiac membrane potential and contractility. [12] Preventing further depolarization of the membrane may avert EKG abnormalities such as QT interval prolongation and STE. [12] Previous studies have shown EETs are potent activators of vascular and cardiac *pm*K_{ATP}. [12-14] Although the mechanism(s) are unknown, evidence suggests that mouse cardiac *pm*K_{ATP} channels are activated by EETs directly inhibiting ATP binding, whereas activation of vascular *pm*K_{ATP} is mediated by a cAMP-protein kinase A (PKA) dependent mechanism.[14] There are various reports demonstrating EET-mediated activation of other cardiac ion channels. For example, cell culture models suggest that EETs inhibit cardiac Na^+ channels [15], modulate Ca^{2+} currents [16, 17] and shorten ventricular APD by activating Kv4.2 channels in a PKA-dependent manner.[18] Together, these data suggest EET-mediated action involves modulation of ion channels.

Recently, we reported that transgenic mice with cardiac-specific over expression of human CYP2J2 or mice with targeted disruption of soluble epoxide hydrolase (sEH null) had increased cardiomyocyte EET biosynthesis, enhanced *pm*K_{ATP} activity and improved postischemic recovery of left ventricular

function.[11, 19, 20] To further examine the cardiac effects of CYP-derived eicosanoids towards ischemia-reperfusion induced EKG abnormalities, we evaluated the role of EETs in an *ex vivo* EKG model. Our initial data show elevated levels of EETs attenuate changes in measures of electrocardiogram parameters (QT interval and STE). Moreover, the data from this chapter suggest that this cardioprotection is mediated by CYP epoxygenase metabolites of AA and involves activation of PKA and *pm*K_{ATP} channels.

3.2 Materials and methods

3.2.1 Animals

Commercially available C57Bl6 mice were purchased from Charles River Laboratories (Charles River Laboratories, Inc.). Mice with cardiac myocyte-specific over expression of human CYP2J2 (CYP2J2 Tr) [11] were obtained from Dr. Darryl Zeldin (NIEHS, RTP, NC, USA). All studies used mice aged 3-4 months, weighing 25-35g. Experiments were conducted in strict guidelines provided by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS).

3.2.2 Ex vivo Cardiac Function and Electrocardiogram Recording

Hearts were perfused in the Langendorff mode, cardiac function measured as left ventricular developed pressure (LVDP), and EKG parameters were assessed as previously described.[11, 21-23] Briefly, hearts from C57Bl/6, CYP2J2 Tr or respective age/sex-matched WT littermate controls were cannulated and perfused in a retrograde fashion at constant pressure ($90cmH_2O$) with continuously aerated ($95\%O_2/5\%CO_2$) Krebs-Henseleit buffer at 37^oC. For assessment of EKG parameters, three electrodes were utilized. The first was placed on the surface of the right atrium, the second on the left ventricle and the third grounded to the apparatus stand. All probes were connected to an amplifier and digital converter for signal recording at the 100 mv range with low pass 1 KHz and high pass 0.3 KHz filters. EKG parameters were manually calculated by measuring the time difference from start of the Q wave to the end of the T wave, and the interval from start of the P wave to the start of the R interval; repeat experiments were

analyzed blinded to the experimenter. Variability in the parameters was reduced by averaging the EKG parameters at 20 sec intervals for 2 min during baseline and at 20min of reperfusion R20. Parameters of EKG recordings were analyzed using ECG module Chart software (ADInstruments). QT interval was corrected (QTc) for heart rate variation using Bazett's equation.[24] Validation of *ex vivo* EKG parameters was assessed by perfusing hearts with halofantrine (+/-) (0, 50 or 100 μM), a kind gift from SmithKline Beecham (Worthing, UK).[25, 26] In the ischemia reperfusion experiments, alterations in EKG parameters were assessed from hearts which were perfused with buffer for a 10min stabilization period, then subjected to 20min global no-flow ischemia, followed by 40min reperfusion (Fig 1). Alterations in contractile function were assessed from hearts which were perfused with buffer for a 40 min stabilization period, then subjected to 20 min global no-flow ischemia, followed by 40 min reperfusion. Recovery of contractile function was taken as left ventricular developed pressure (LVDP) at the end of reperfusion expressed as a percentage of pre-ischemic LVDP (Fig. 1). Lactate dehydrogenase (LDH) levels were assessed in heart perfusate collected during EKG analysis using a commercially available kit (Sigma-Aldrich, Oakville, ON).

For some experiments, hearts were perfused with either the selective P450 epoxygenase inhibitor MS-PPOH (50μM), EET antagonist 14,15-EEZE(10μM), 11,12-EET (+/-) (1μM), 14,15-EET (+/-) (0.5, 1 or 3μM), the *pm*K_{ATP} channel inhibitor glibenclamide (GLIB, 1μM, Sigma-Aldrich, Oakville, ON), the selective *pm*K_{ATP} channel inhibitor HMR1098 (10μM, Aventis Pharmaceuticals, Frankfurt, Germany), the PKA inhibitor H89 (50nM, Sigma-Aldrich, Oakville,

ON) or vehicle administered immediately after 20 min ischemia and for the duration of the 40 min reperfusion period.

3.2.3 Immunoblotting

Protein was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted as previously described.[11, 19] Cytosolic and plasma membrane fractions were prepared from frozen mouse hearts as described.[27] Briefly, hearts were homogenized in buffer containing (sucrose 250mM, TrisHCL 10mM, EDTA 1mM, sodium orthovanadate 1mM, sodium flouride 1mM, aproptinin 10µl/L, leupeptin 2µl/L, pepstatin 100µl/L) and centrifuged at 10,000xg for 20 min. Pellets were resuspended in homogenisation buffer and centrifuged at 750xg for 10 min. Supernatant was separated and pellets were resuspended in homogenisation buffer and again centrifuged at 270g for 10 min, repeated two times. All supernatant fractions collected were pooled together and centrifuged at 10,000xg for 30 min. The subsequent supernatant was then centrifuged at 48,000xg for 60 min to separate plasma membrane (PM pellet) and cytosolic (supernatant) fractions. PM pellets were subsequently resuspended in homogenization buffer. Protein quantities were determined using a Bradford protein assay kit (BioRad Laboratories, Canada). Immunoblots were prepared using cytosolic (30µg protein) or plasma membrane (15µg protein) fractions and probed with antibodies to total and phospho-PKAαII (regulatory) 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA) and Kir6.2 (Santa Cruz Biotechnology, Santa Cruz, CA). Relative band intensities, expressed in arbitrary units of

phospho-PKAαII (regulatory) to total PKAαII (regulatory), were assessed by densitometry using Image J (NIH, USA).

3.2.4 Statistical analysis

Values are expressed as the mean ± SEM. Statistical significance was determined by the unpaired Student's *t*- test and one-way Duncan's tests were performed to assess differences between groups. Values were considered significant if $p < 0.05$.

3.3 Results

3.3.1 Characterization

EKG parameters obtained from the *ex vivo* model were first characterized by recording changes in C57BL/6 hearts perfused with halofantrine (+/-). Isolated hearts were first perfused for 10 min to obtain stable baseline readings, and then subsequently perfused with different concentrations of halofantrine (+/-) (0, 50 or 100μM, Fig 3-1A, 3-2A). Prolongation of QTc intervals were observed following increasing concentrations of halofantrine (Fig 3-2A). These data indicated our *ex vivo* model was able to assess EKG parameters in a predictable manner, and moreover, was consistent with previously published data [25].

3.3.2 Ventricular Repolarization Following Ischemia Reperfusion in Perfused Hearts

To investigate the role of eicosanoids in improving ventricular repolarization after ischemic injury two established models of elevated EETs were utilized; one, C57BL/6 mice perfused with physiological concentrations of exogenous 14,15-EET (0.5, 1 or 3μM) or 11,12-EET (1μM) and transgenic mice with the cardiomyocyte specific overexpression of human CYP2J2 [11, 19]. Each model was assessed for alterations in EKG parameters and LVDP. Baseline characterization of EKG parameters showed no significant differences in any model compared to their respective controls (Fig 3-1 and 3-3). Hearts from C57Bl/6 mice perfused with either 11,12-EET or 14,15-EET did not have significant prolongation in postischemic QTc intervals (<10% of baseline) whereas significant prolongation was observed in vehicle perfused hearts (>40%

of baseline) (Fig. 2B). This improved postischemic outcome was dose dependent with no protective effect observed at the lowest dose of 14, 15-EET (0.5μM) (>30% prolongation relative to baseline) (Fig. 3-2B). CYP2J2 transgenic mice also demonstrated improved postischemic QTc intervals compared to WT mice (<10% prolongation of QTc) (Fig. 3-3A). Consistently, both CYP2J2 Tr hearts and C57Bl/6 mice perfused with 11,12- or 14,15-EET (1μM) did not develop large increases in STE (Table 3-1).

To determine if the improved postischemic ventricular repolarization in CYP2J2 Tr hearts was mediated by a P450 epoxygenase metabolite, we conducted experiments in the presence of (50μM) MS-PPOH [11]. The epoxygenase inhibitor caused a further but non-statistically significant increase in QTc prolongation in WT mice (Fig. 3-3A). Importantly, MS-PPOH completely abolished the improved postischemic recovery in CYP2J2 Tr mice (>400% prolongation of QTc compared to control) (Fig. 3-3A). To further demonstrate that the protective effect was mediated by EET's, hearts from C57Bl/6 mice perfused with 14,15-EET (1 μM) were treated with the EET antagonist, 14,15-EEZE (10 μM) during reperfusion. The antagonist caused a significant increase in postischemic QTc in hearts treated with EET (>500% QTc prolongation compared to EET-treated) and further increased postischemic QTc and STE in WT animals (Table 3-1, Fig. 3-3B). These data indicate that the postischemic cardioprotective effect is mediated by CYP epoxygenase metabolites and further suggest a role of endogenous EETs in WT mice.

3.3.3 Role of *pm*K_{ATP} in Postischemic EKG

To examine whether the improved ventricular repolarization in animals with elevated levels of EETs was due to activation of *pm*K_{ATP} channels, experiments were conducted in the presence of the non-specific *pm*K_{ATP} channel blocker glibenclamide (1μM) or the selective *pm*K_{ATP} inhibitor HMR1098 (10μM) [14, 20]. Inhibitors were perfused at the time of reperfusion and remained present for the duration of the protocol. Perfusion with either GLIB or HMR1098 resulted in a small but non-significant further prolongation of QTC and STE in WT hearts (Table 3-1, Fig. 3-4). Interestingly, GLIB abolished the beneficial effect of EETs on QTc prolongation and STE in C57Bl/6 hearts (N250% QTc prolongation compares to EET-treated) (Table 3-1, Fig.3-4). In addition, both GLIB and HMR1098 caused significant QTc prolongation and STE in CYP2J2Tr hearts (>250% compared to controls) (Table 3-1, Fig 3-5). No differences in Kir6.2 subunit expression were observed between CYP2J2 and WT (Fig 3-7B). Together these data suggest the involvement of *pm*K_{ATP} in the cardioprotective effect of EETs.

3.3.4 Functional Recovery after Ischemia-Reperfusion in Perfused Hearts

Hearts from CYP2J2 Tr mice or C57Bl/6 mice perfused with 11,12-EET (1μM) had significantly improved postischemic recovery of left ventricular function compared to respective controls (Fig 3-5A, 5B). The improved function was evident within 20 min of reperfusion and persisted throughout the recovery period (Fig 3-5A, 5B). At 40 min reflow, LVDP recovery was significantly higher in EET-treated and CYP2J2 Tr (11, 12-EET, 71±16%; CYP2J2 Tr,

51±3%) than WT hearts (30±4%) (Fig.3-5A, 5B). There were no statistically significant differences between WT, 11,12-EET treated or CYP2J2 Tr hearts in time-to-onset of ischemic contracture (12.1±1 min, 13.8±1 min vs. 13.4±1min, respectively) or maximal ischemic contracture (53±7 cmH_20, 45±6 cmH_20 vs. 65±3 cmH_20, respectively). Consistent with these results, a marked reduction in LDH levels was found in heart perfusate from C57Bl/6 mice treated with 11, 12-EET compared to vehicle controls during reperfusion (Fig 3-5C). To more specifically examine the role of *pm*K_{ATP} channels in the cardioprotective phenotype of CYP2J2 Tr mice, the specific *pm*K_{ATP} inhibitor HMR1098 (10 μM) was added to perfusion buffer at the time of reperfusion. Its administration abolished the postischemic functional recovery observed in the CYP2J2 Tr mice (Fig 3-5A, 5B).

3.3.5 EET Mediated Protection and PKA

Evidence in the literature suggests activation of ion channels are related to increased intracellular cAMP production and PKA dependent phosphorylation [14, 17, 28, 29]. In order to further investigate the effect of EETs on improved postischemic ventricular repolarization, we examine the role of PKA. To determine if activation of PKA was required for EET-mediated cardioprotection, we administered the PKA inhibitor H89 (50nM) during reperfusion in C57Bl/6 mice treated with vehicle or 14, 15-EET and in CYP2J2 Tr hearts. Interestingly, treatment with H89 significantly increased postischemic QTc and STE in CYP2J2 Tr hearts (>300%) and in EET-treated hearts (>250%) but had minimal effect on vehicle treated hearts (Table 3-1, Fig 3-6). Further analysis of PKA protein

subunits in CYP2J2 Tr and WT mice, following ischemia-reperfusion, demonstrated no differences in total-PKAα regulatory-II unit expression in the cytosol and plasma membrane fractions or subcellular fractions (Fig 3-7). Interestingly, increased expression of phosphorylated PKAα regulatory-II subunit was found in plasma membrane of hearts from CYP2J2 Tr mice but not in the cytosolic fractions (Fig 3-7). Expression of Kir6.2 subunit confirmed membrane fractions were isolated (Fig 3-7) and thus suggests that EET mediated activation is partially due to enhanced phosphorylation of PKAα regulatory-II subunit co-localized to membrane fractions.

3.4 Discussion

While evidence has begun to identify the significance of cardiac cytochrome P450s to heart function and protection, effects are dependent on the metabolites produced.[11, 19, 30-34] CYP epoxygenases (EETs) have well established cardioprotective effects within the cardiovascular system [11, 35], whereas metabolites from CYP ω-hydroxylases (20-HETE) can be detrimental. CYP epoxygenase-derived eicosanoids can affect cardiomyocyte function [15, 16, 36] and improve postischemic functional recovery. [11, 19, 30, 34, 36] Recent data has shown that deletion of *Ephx2* in mice provides anti-arrhythmic effects towards atrial and ventricular arrhythmias in a model of heart failure [37] and reduced risk of infarction in the hearts perfused with CYP epoxygenase metabolites. [38, 39] The data presented here in this chapter provide further direct evidence that EET-mediated cardioprotective response is important following ischemic injury. We have demonstrated that EETs play a significant role in ventricular repolarization following postischemic recovery in isolated mouse hearts. Utilizing two models to elevate cardiac EETs; C57Bl/6 mice treated with exogenous EETs and CYP2J2 Tr mice, we show improved ventricular repolarization after ischemic injury compared to respective WT mice. Moreover, the potent EET antagonist 14,15-EEZE and epoxygenase inhibitor MS-PPOH reversed the cardioprotective response in the EET treated and CYP2J2 Tr groups, implicating EETs in this process.[38] In addition, our results demonstrate an important role for *pm*K_{ATP} channels and PKA-mediated signalling in the protective mechanism. Taken

together, these data provide more evidence that EETs have important functional and electrophysiological effects in the ischemic heart.

In order to validate the data acquired in our *ex vivo* EKG model of ischemia/reperfusion, parameters were first characterized by perfusing hearts with a drug known to cause abnormalities.[21, 25, 26] Halofantrine, an antimalarial drug known to cause prolongation of QT interval at high concentrations, triggers QT prolongation by blocking the delayed rectified potassium channels.[25] When isolated hearts were perfused with a racemic mix of halofantrine, a predicted dose dependent prolongation of QT interval was observed. [25, 26] In conjunction with baseline EKG parameters, the predicted halofantrine response demonstrated in the *ex vivo* model was capable of both reproducing known EKG abnormalities and providing reliable information.

Ischemic events affect repolarization over the ventricular myocardium resulting in prolonged QT intervals and STE.[6, 40-42] These abnormalities can lead to ventricular arrhythmias or sudden cardiac death. The underlying electrophysiological action leading to QT interval prolongation and STE is not fully elucidated. However, the delayed and non-uniform recovery of repolarization is partially attributed to changes in ionic imbalances of Ca^{2+}, K^{+}, H^{+} and Na^{+} leading to alteration in the repolarization currents. [42-45] Such changes in intracellular currents flowing to and from the ischemic region may effectively prolong APD leading to QT prolongation and STE. Previous work from our CYP2J2 transgenic mice has demonstrated that K^{+} channels are significantly enhanced, similar to data from Gross *et al* [11, 18-20, 34, 46] and consistent with

improved rates of ventricular contraction (dP/dt_{max}) and relaxation ($-dP/dt_{min}$) following ischemia. [11, 19, 20] EETs have been shown to be potent openers of *pm*K_{ATP} by reducing channel sensitivity to ATP in isolated rat myocytes using the inside-out patch clamp technique; however, the exact site on the channel that interacts with EETs remains unknown.[12, 13, 18] The current results demonstrate that baseline EKG parameters were similar in all groups and significant prolongation in QT interval and STE occurred to a greater extent in WT mice following the ischemic event, compared to EET-treated or CYP2J2 Tr mice. Moreover, the reduction in abnormal EKG waveforms observed in CYP2J2 Tr or EET-perfused hearts correlated with reduced injury and improved postischemic cardiac function. These protective effects were inhibited by blocking K_{ATP} channels, specifically the *pm*K_{ATP} channel. Involvement of *pm*K_{ATP} channels in ischemia/reperfusion injury has been demonstrated in the Kir6.2 knockout mouse where ischemic preconditioning could not be produced.[47] Activation of *pm*K_{ATP} channels can protect the heart against ischemic reperfusion injury by hyperpolarizing the cell, limiting calcium entry and preserving ATP utilization, thereby maintaining cardiac membrane potential and contractility.[46, 48] Stopping the further depolarization of the membrane protects the heart against injury and prevents QT interval prolongation and STE. This is accomplished, in part, by increasing the outward K^+ current thereby enhancing ventricular repolarization.[46, 48, 49] Our data from western blotting showing no difference in expression of *pm*K_{ATP} subunit Kir 6.2, suggest the protective effect results from activation of the channel and not differences in subunit protein expression.

Previous data suggested that EET-mediated protection involves activating mitoK_{ATP} channels. However, considering that the identity of the mitoK_{ATP} channel has yet to be determined, it is unknown whether EETs directly target these channels or work through upstream signals. The current results suggest the notion that postischemic EET-mediated protection begins upstream of mitoK_{ATP} channels, potentially targeting *pm*K_{ATP}. Recent evidence indicating the cardioprotective effects of the EET antagonist, 14, 15-EEZE, targets a receptor or protein upstream of the mitoK_{ATP} channel provides support for this protective pathway. [38, 50] Interestingly, Marinovic *et al* recently demonstrated that activation of *pm*K_{ATP} during hypoxic stress prevents mitochondrial damage which suggests an intracellular connection between the plasma membrane channel and mitochondria. [51] While the precise mechanisms or relative contribution of each channel to improved recovery is unclear, EET-mediated protective effects appear to be complex.

The improved recovery observed in mice treated with 14, 15-EET and CYP2J2 Tr was attenuated with H89, a PKA inhibitor. Supporting this functional data, increased plasma membrane expression of activated PKAα regulatory II subunit was observed in CYP2J2 Tr hearts. Expression of activated PKAα regulatory II subunit in the plasma membrane fraction suggests the involvement of PKA in ion channel activity. [28] While activation of vascular *pm*K_{ATP} channels by EETs occurs via cAMP-PKA mediated event [14]; cell culture experiments suggest EET-mediated activation of mouse cardiac *pm*K_{ATP} channel, occurs in a voltage dependent manner. [18] However, EETs are also known to activate other

ion channels through modulation of intracellular cAMP and PKA protein levels. [14, 17] Recently, Ke *et al* [18] demonstrated that CYP2J2-derived EETs affect cardiac APD and $I_{to,peak}$ via a cAMP-dependent mechanism. It was suggested that EETs increase intracellular cAMP production triggering a cAMP-PKA dependent phosphorylation of Kv4.2 channels resulting in enhanced maximal peak transient outward currents. The fact that inhibitors of either pathway completely abolish the improved postischemic ventricular repolarization suggests a potential role in EET-mediated cardioprotection.

In summary, the data from this chapter demonstrate that EETs improve postischemic ventricular repolarization and attenuate electrocardiogram abnormalities, such as QTc prolongation and STE, which corresponded to improved functional recovery and reduced injury. The results suggest this occurs potentially via activation of *pm*K_{ATP} channels and a PKA-dependent signal. Taken together, the data are consistent with an important modulatory effect of CYP-derived eicosanoids in cardioprotection which contribute to improved recovery of heart contractile function and electrophysiological abnormalities after global ischemia.

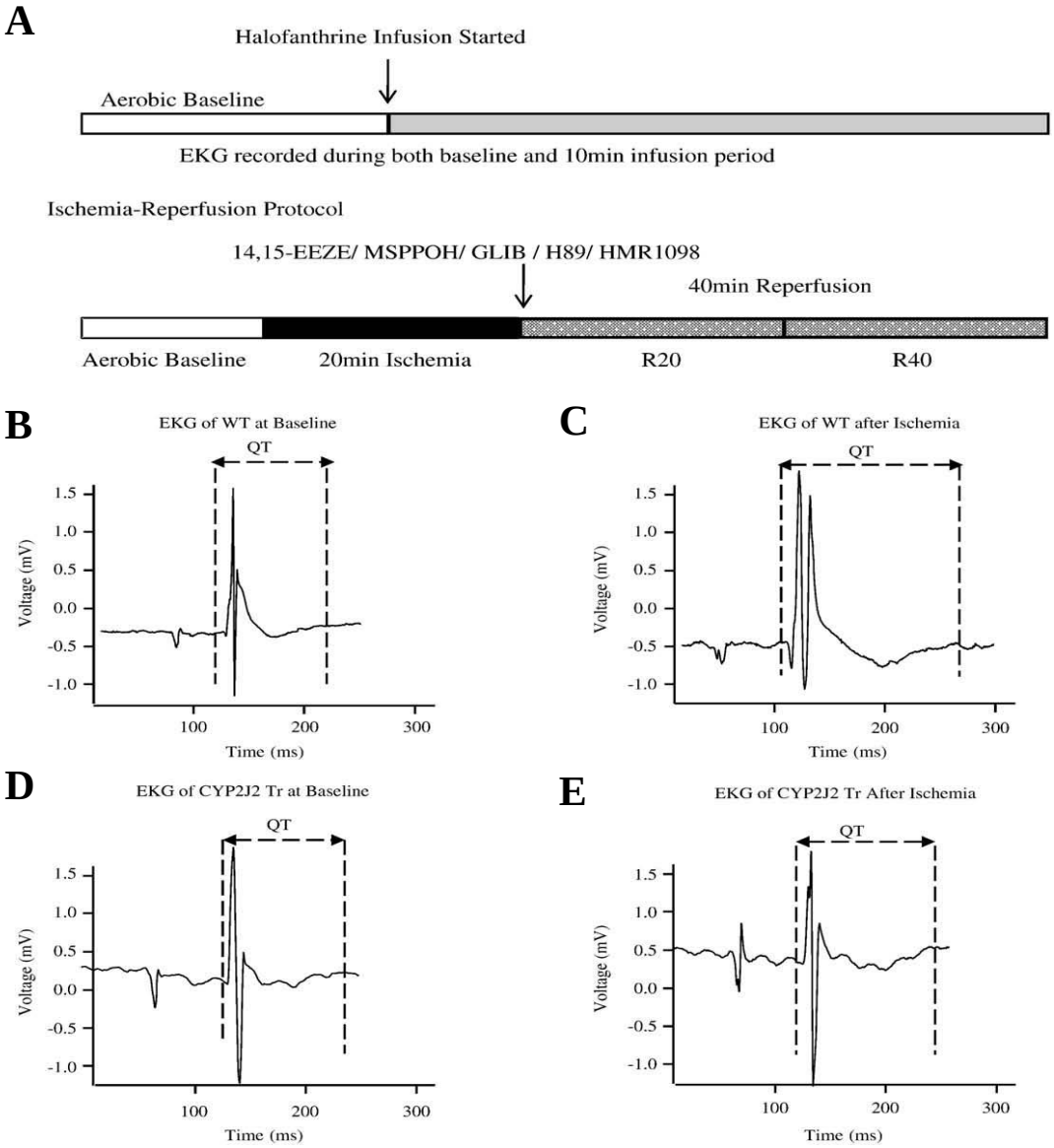

Fig.3.1 Perfusion protocol of *ex vivo* electrocardiogram parameters and representative EKG tracings. (A) Diagram showing experimental protocol with the time courses of drugs perfused and EKG and LVDP recording. (B) EKG tracing from a C57Bl/6 heart showing QT interval at baseline. (C) EKG tracing from a C57Bl/6 heart showing the QT interval prolongation 20 min after ischemia. (D) EKG tracing from a CYP2J2 transgenic heart showing QT interval at baseline. (E) EKG tracing from a CYP2J2 transgenic heart 20 min after ischemia.

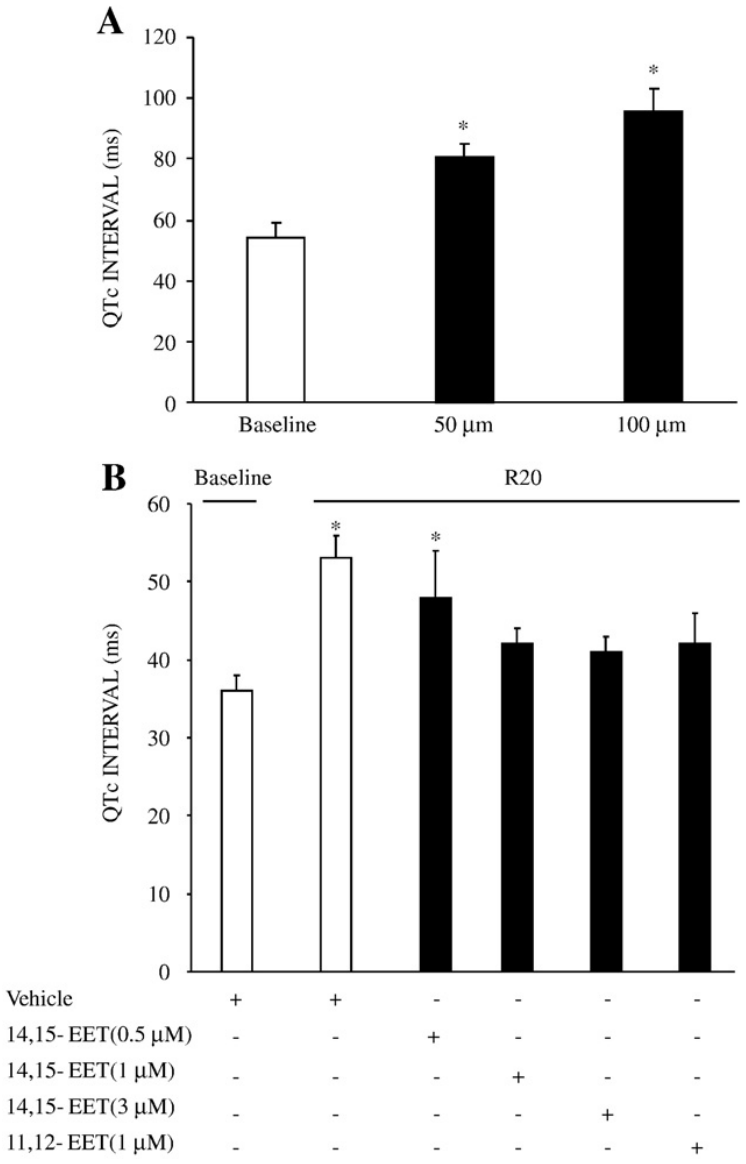

Fig.3.2 (A) Histogram of QTc interval prolongation with increasing halofantrine (+/−) concentrations. Values represent mean±SEM, n=3–5; *, $P<0.05$ vs. baseline. (B) Histogram of QTc interval changes in hearts from C57Bl/6 mice perfused with vehicle, 14, 15-EET (0, 0.5, 1 or 3 µM) or 11, 12-EET (1 µM) at baseline and 20 min following global ischemia. Values represent mean±SEM, n=3–10; *, $P<0.05$ vs. baseline of vehicle control.

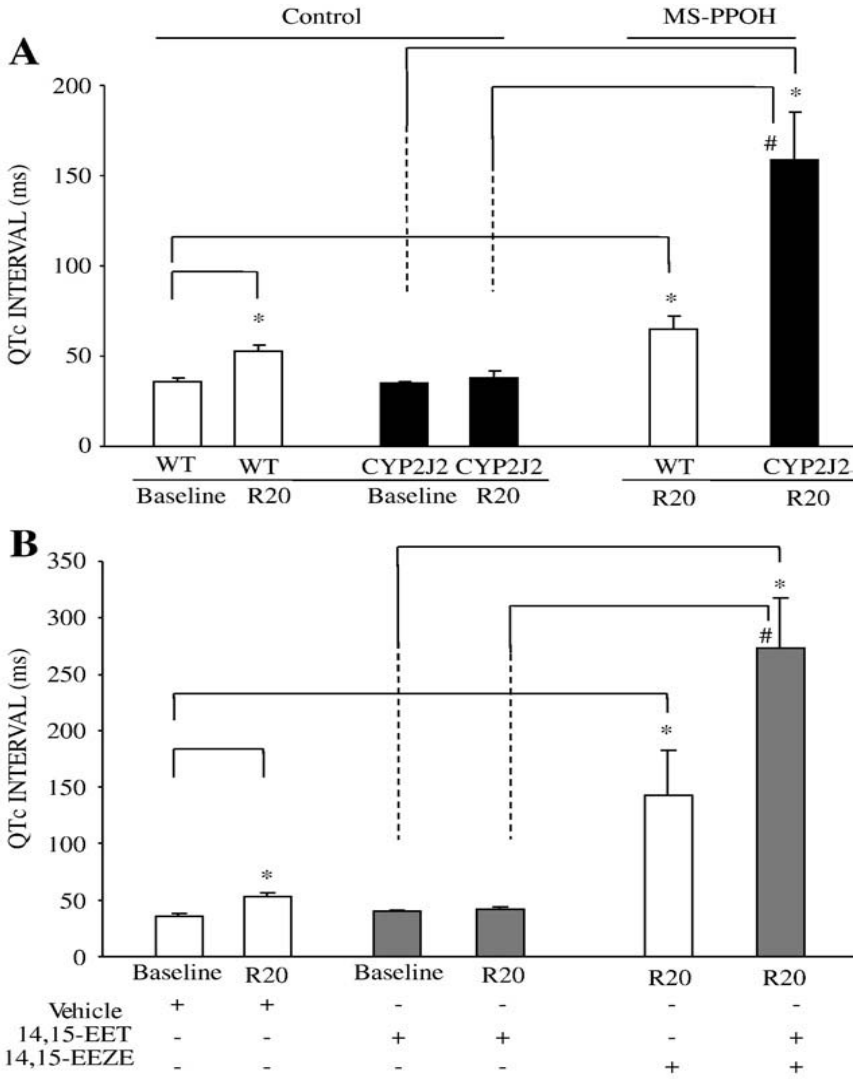

Fig.3.3 (A) Histogram of QTc interval changes in hearts from CYP2J2 Tr and WT littermate mice perfused with vehicle or MSPPOH (50 μM) at baseline and 20 min following global ischemia. Values represent mean±SEM, n=4–10;*. *P*<0.05 vs. vehicle control; #, *P*<0.05 vs. R20 of vehicle control of same genotype. (B) Histogram of QTc interval changes in hearts from C57Bl/6 mice perfused with vehicle, 14, 15-EET (1 μM) or 14, 15-EEZE (10 μM) at baseline and 20 min following global ischemia. Values represent mean±SEM, n=4–7;*. *P*<0.05 0.05 vs. baseline of vehicle control; #, *P*<0.05 vs. R20 of 14, 15-EET perfused hearts.

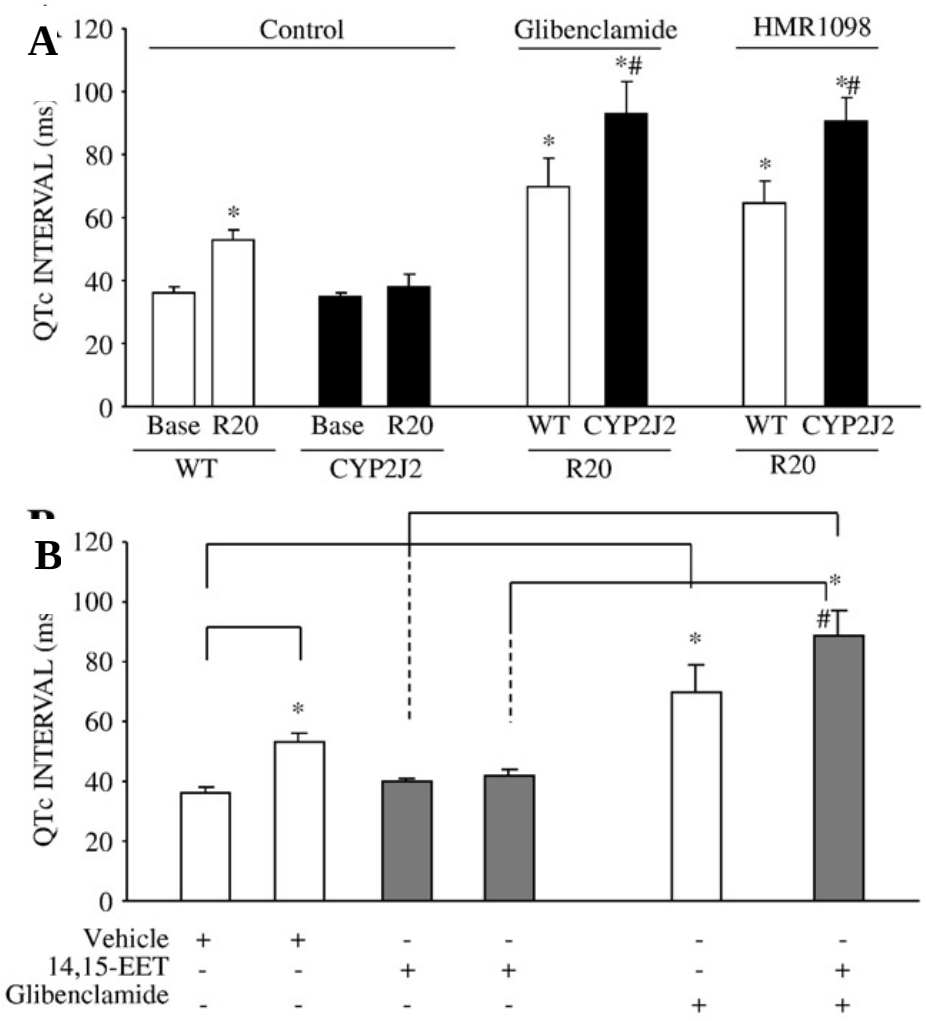

Fig.3.4 (A) Histogram of QTc interval changes in hearts from WT littermates and CYP2J2 Tr mice perfused with vehicle, GLIB (1 μM) or HMR1098 (10 μM) at baseline and 20 min following global ischemia. Values represent mean±SEM, n=4–7; *. *P*<0.05 vs. baseline of vehicle control; #, *P*<0.05 vs. R20 of vehicle control of same genotype. (B) Histogram of QTc interval changes in vehicle and GLIB (1 μM) perfused hearts from C57Bl/6 mice treated with 14,15-EET (1 μM) at baseline and 20 min following global ischemia. Values represent mean±SEM, n=4–7; *. *P*<0.05 vs. baseline of vehicle control; #, *P*<0.05 vs.R20 of 14, 15-EET perfused hearts.

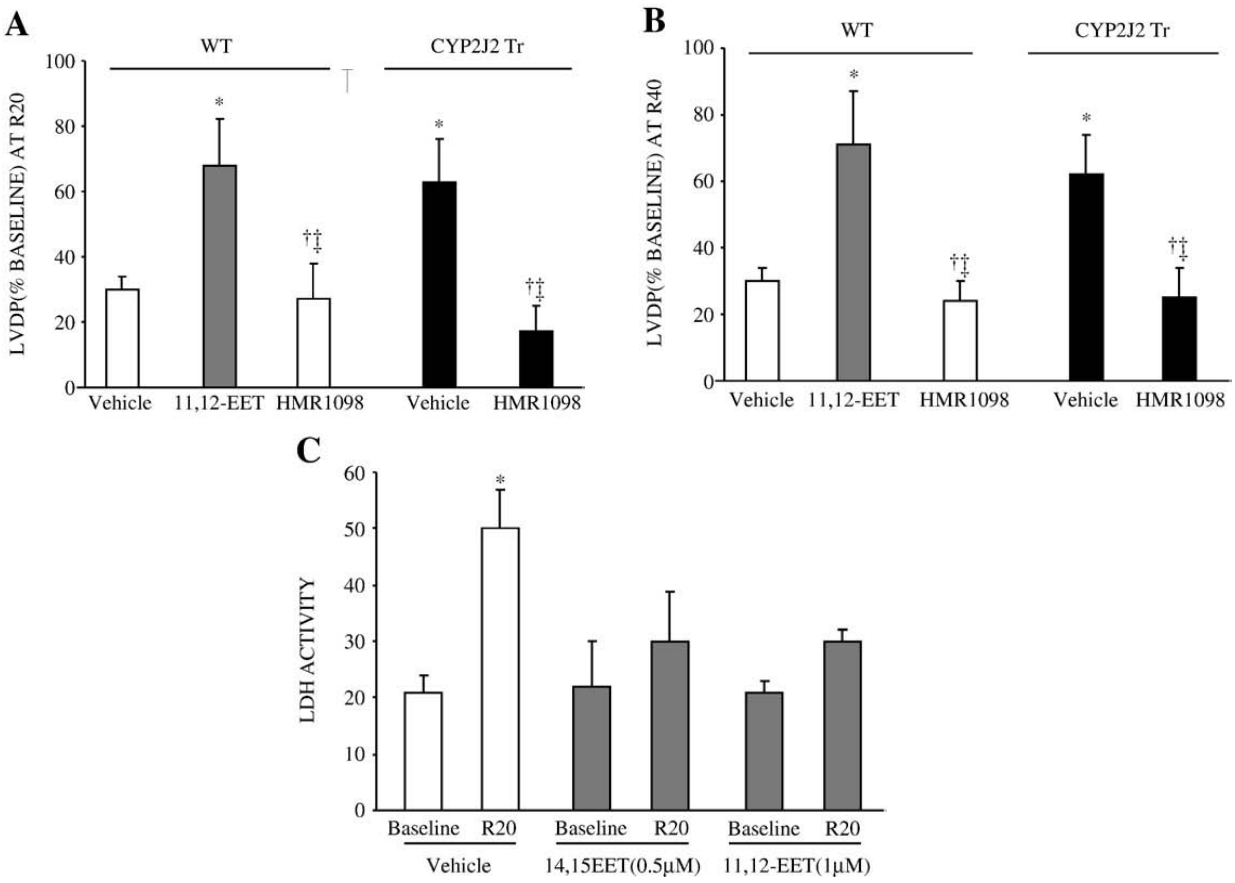

Fig.3.5 Postischemic recovery of left ventricular function and LDH activity following ischemia reperfusion. Postischemic recovery of left ventricular function in hearts from CYP2J2 Tr and C57Bl/6 mice perfused with 11,12-EET (1 μM) or HMR1098 (10 μM). (A) Functional recovery at 20 min reperfusion expressed as a percentage of baseline LVDP. (B) Functional recovery at 40 min reperfusion expressed as a percentage of baseline LVDP. Values represent mean±SEM, n=5–10; *, $P<0.05$ vs. WT vehicle control; †, $P<0.05$ vs. 11, 12-EET; ‡, $P<0.05$ vs. CYP2J2 Tr control. (C) LDH activity, as arbitrary units, in perfusate from C57Bl/6 mice hearts perfused with vehicle or 14, 15-EET (0.5 μM) or 11, 12-EET (1 μM). Values represent mean±SEM, n=4; *, $P<0.05$, vs. vehicle control.

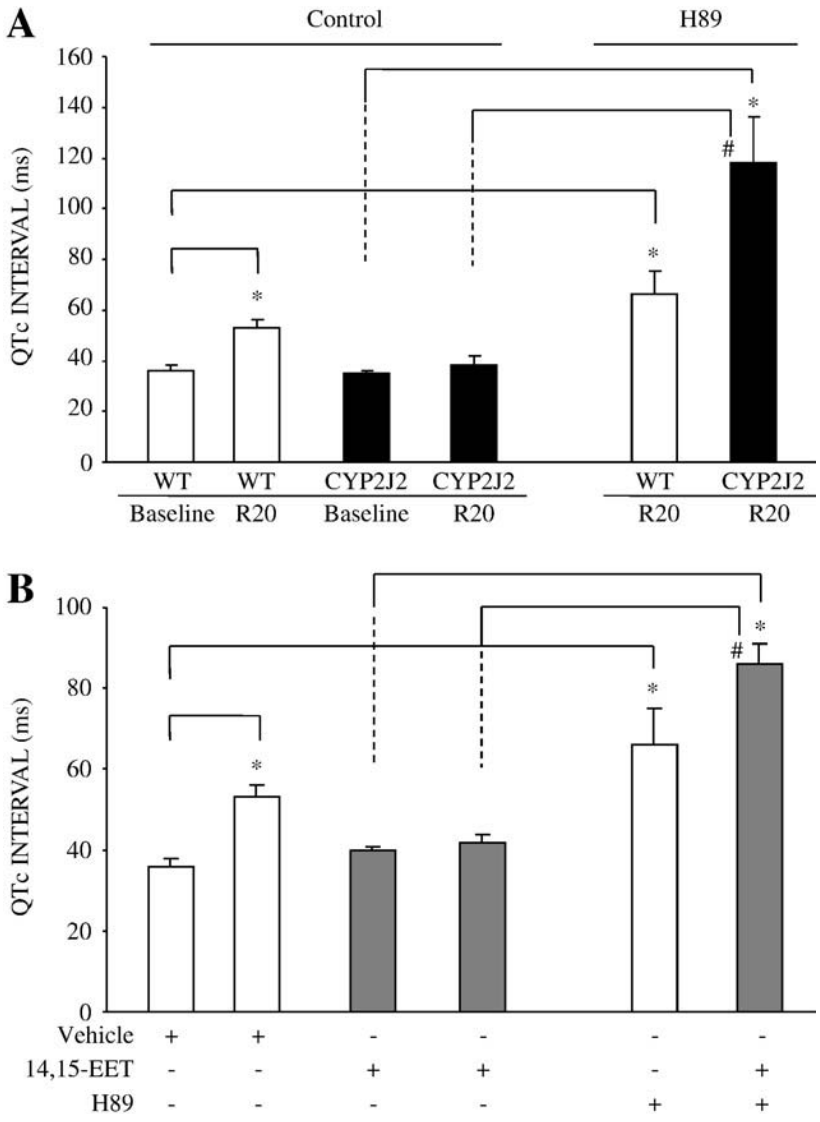

Fig.3.6 (A) Histogram of QTc interval changes in vehicle and H89 (50 nM) perfused hearts from WT littermates and CYP2J2 Tr mice at baseline and 20 min following global ischemia. Values represent mean±SEM, n=4; *, $P<0.05$ vs. baseline of vehicle control; #, $P<0.05$ vs. R20 of vehicle control. (B) Histogram of QTc interval changes in vehicle and H89 (50 nM) perfused hearts from C57Bl/6 mice treated with 14, 15-EET (1 μM) at baseline and 20 min following global ischemia. Values represent mean±SEM, n=4–7; *, $P<0.05$ vs. baseline of vehicle control; #, $P<0.05$ vs. R20 of 14, 15-EET perfused hearts.

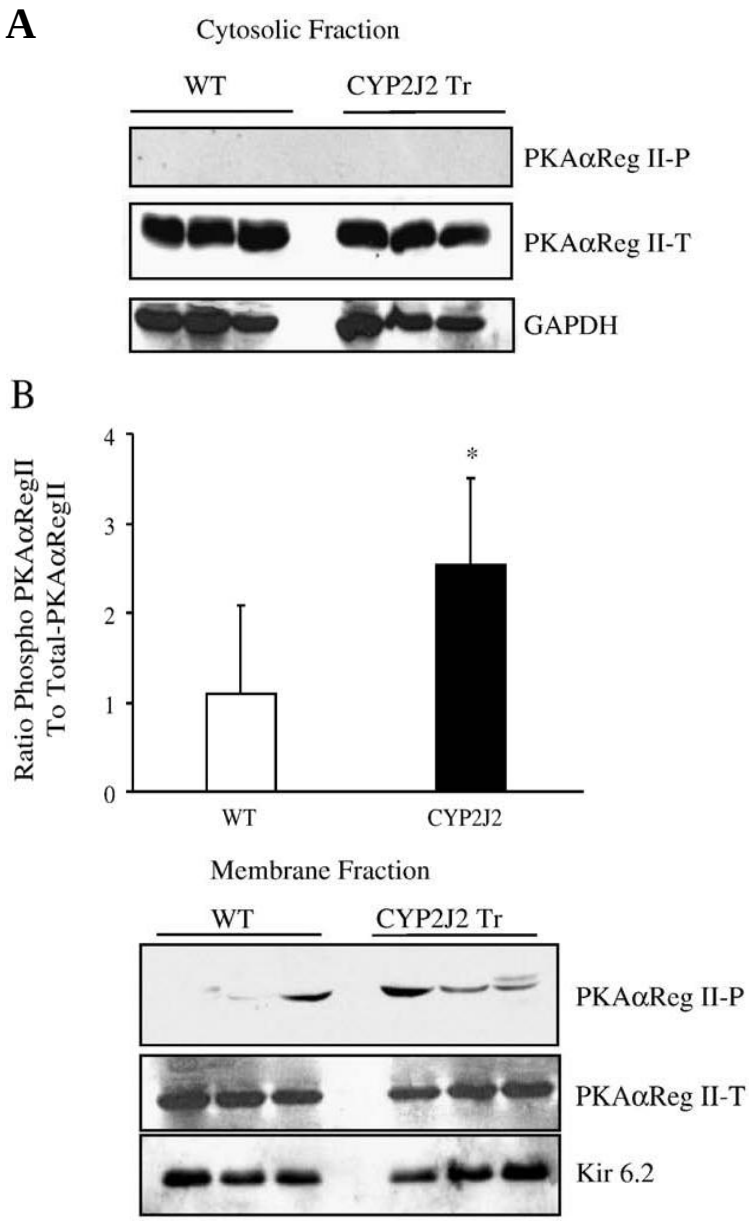

Fig.3.7 PKA role in EET-mediated ventricular repolarization. (A) Representative immunoblots showing cytosolic and plasma membrane expression of total PKAα regulatory II, phospho-PKAα regulatory II and Kir6.2 at 40 min following global ischemia. (B) Represents the densitometry ratio of phospho-PKAα regulatory II to total PKAα regulatory II expression after 40 min reperfusion following treatment. Values represent mean±SEM, n=5; *, $P<0.05$.

ST Elevation (mv)	WT	CYP2J2 Tr	14,15-EET
Baseline	2.3±0.2	1.9±0.1	2.2±0.3
Reperfusion			
Control	3.6±0.4*	1.3±0.2	1.2±0.2
MS-PPOH (50μM)	- - - -	3.0±0.9*†	- - - -
14,15- EEZE (10μM)	3.5±0.7*	- - - -	2.7±0.4†
Glibenclamide (1μM)	3.5±0.5*	3.0±0.2*†	3.4±0.5*†
HMR1098 (10μM)	3.5±0.4*	4.0±1.3*†	- - - -
H89 (50nM)	3.5±0.3*	2.4±0.1†	3.9±0.7* †

Table 3.1 ST elevation was assessed during baseline (preischemic) and after 20 min of reperfusion (R20) in WT, CYP2J2 Tr and C57Bl/6 mice treated with 14, 15-EET (1μM). Hearts were perfused with vehicle (control), MS-PPOH (50μM), 14, 15-EEZE (10μM), glibenclamide (1μM), HMR1098 (10μM) or H89 (50nM) as outlined. Values represent mean±SEM, n=5–10.

*, $p<0.05$ vs. baseline control of same genotype.

†, $p<0.05$ vs. control R20 STE of same genotype.

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CHAPTER-IV

Cardioprotective effect of a dual acting epoxyeicosatrienoic acid analogue towards ischemia reperfusion injury[4]

[4]*A version of this chapter has been published: Batchu SN, Lee SB, Qadh RS, Chaudhary KR, El-Sikhry H, Kodela R, Falck JR, Seubert JM. Cardioprotective effect of a dual acting epoxyeicosatrienoic acid analogue towards ischemia reperfusion injury. British Journal of Pharmacology 2011 Feb; 162(4): 897–907*

4.1 Introduction

Arachidonic acid (AA) metabolites generated by cytochrome P450 epoxygenases (CYP), epoxyeicosatrienoic acids (EETs), are important lipid mediators involved in regulating cardiac function and protection against ischemia reperfusion injury. EETs exist as four regioisomers (5, 6-; 8, 9-; 11, 12- and 14, 15-EET) that are rapidly converted to the corresponding and less biologically active dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). [20, 22] EET-mediated actions can be reduced by metabolism via beta-oxidation, esterification and autooxidation [6. 10,29,] Elevation of intracellular EETs has been shown to have cardioprotective effects against ischemic-reperfusion (IR) injury. Although the precise mechanism(s) remains elusive, studies have demonstrate that the protective mechanism(s) involves modulation of ion channels such as the ATP-sensitive potassium channel (K_{ATP}) and signalling through phosphoinositide 3-kinase (PI3K) pathways targeting the mitochondria.[7, 15,19,28,29]

EETs are chemically and metabolically labile; a rapid metabolism, low solubility and storage issues limit their use as therapeutic agents and pharmacological tools. [18] Indeed, difficulty arises with long-term treatment using EETs because they are rapidly metabolized or incorporated into membranes. [32]As such, considerable interest has arisen in developing methods to enhance the bioavailability of EETs. To overcome these limitations, the administration of pharmacological inhibitors of CYP epoxygenases (MSSPOH) [28] and sEH (*t*AUCB) [4] have been utilized. However, current sEH inhibitors are limited in effect as they rely on endogenous EET production, which undergoes further rapid metabolism and/or incorporation into membranes.

Recently developed EET-analogues possessing EET-mimetic and sEH inhibitory properties contain several key features: (i) a partially saturated carbon backbone to avoid autooxidation and improve physical stability, (ii) a *cis*-$\Delta^{8,9}$-olefin for EET-mimetic activity, and (iii) 1,3-disubstituted urea for sEH inhibitory properties that prolongs the half-life (Fig. 1A) [9]. In the present chapter, we report cardioprotective effects of the novel EET analog, UA-8. Moreover, our data demonstrate marked reduction in infarct size and reduced mitochondrial damage at nanomolar concentrations that involves a Class-I PI3K-dependent pathway

4.2 Materials and Methods

4.2.1 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. C57BL/6 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 C57BL/6 genetic background for more than 7 generations, is maintained at the University of Alberta.

4.2.2 Isolated heart perfusion

Hearts were perfused in the Langendorff mode as described.[2, 5] Briefly, hearts from mice were perfused in a retrograde fashion at constant pressure (90cmH_2O) with continuously aerated (95%O_2/5%CO_2) Krebs-Henseleit buffer at 37^oC. Hearts were perfused for 40 min (stabilization), and then subjected to 20 min of global no-flow ischemia, followed by 40 min reperfusion. Recovery of contractile function was measured as the recovery of LVDP at 40 min of reperfusion and expressed as a percent of preischemic LVDP. For some experiments, hearts were perfused with 11,12-EET (1μM), UA-8 (chemical name: 13-(3-**p**ropyl**u**reido)**t**ridec-8-enoic **a**cid (0.01-1μM), UA-7 (chemical name: 13-(2-(butylamino)-2-oxoacetamido)tridec-8(Z)-enoic acid) (1μM), 14,15-EEZE (10μM), MS-PPOH (50μM), wortmannin (1μM, Sigma-Aldrich, Canada) and PI-103 (1μM, Cayman Chemicals, USA) (Fig. 1A). In all the experiments, hearts were stabilized for 40 min and then subjected to 20 min of ischemia followed by 40 min of

reperfusion (Fig. 1B). To determine the amount of infarction, after 2 h reperfusion following 20 min ischemia, hearts were incubated with a 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 and compared to total area of the slice.

4.2.3 Immunoblotting

Crude cytosolic fractions were prepared from frozen mouse hearts as previously described. [2, 5] Protein was resolved on 12%SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies to phospho- and total-Akt or phospho- and total-GSK3ß (1:1000, Cell Signalling Technology, Inc. USA). Relative band intensities were expressed in arbitrary units assessed using Image J software (NIH, USA).

4.2.4 Cell Culture Experiments

H9c2 cells (ATTC, Manassas, VA) were cultured under anoxic conditions (37°C in an atmosphere of 5% CO_2/ 95% air). DMEM media with phenol red was supplemented with 10% fetal bovine serum, and antibiotics penicillin, streptomycin and amikacin. In all the experiments, cells were treated with 11, 12-EET(1µM) or UA-8(0.1µM). Anoxic conditions were generated in an air tight chamber (MIC101, Billups-Rothenberg, Inc.) and maintained for 6 h followed by 16 h of reoxygenation (Fig. 1B).

Cell viability was assessed by using trypan blue dye exclusion assay. Following anoxia–reoxygenation cells were collected and centrifuged. The

supernatant was discarded and the resultant pellet was resuspended in homogenization buffer as previously described. [5, 7, 19, 29] A 50 μl aliquot was mixed with 0.4% trypan blue dye (Sigma-Aldrich, Oakville, ON) in a 1:1 ratio and allowed to incubate for 3 min. This mixture was then placed on the hemocytometer and viable (unstained) and dead (blue-stained) cells were counted.

Caspase-3 activity was assessed using a spectrofluorometric assay as described previously.[38] Briefly, caspase-3 activity was determined in cytosolic fractions by monitoring the release of 7-amino-4-methylcoumarin (AMC) by proteolytic cleavage of the peptide Ac-DEVD-AMC (20μM) (Sigma-Aldrich, Oakville, ON). Fluorescence was monitored at wavelengths of 380 nm (excitation) and 460 nm (emission). Specific activities were determined to be within the linear range of a standard curve established with AMC.

4.2.5 Mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was studied by loading H9c2 cells with 150nM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen, USA) to record changes in $\Delta\Psi_M$. TMRE is a cationic dye attracted to polarized mitochondria membranes, a reflection of mitochondrial function. Cells were incubated with 150 nM TMRE for 20 min after 6 h anoxia and 16 h reoxygenation. Changes in fluorescence were recorded over a 10 minute period at 37ºC using a Zeiss Axio Observer Z1 inverted epifluorescence microscope to take z-stack images for two channels every 10 sec with a 500 ms exposure time. Cells were observed under a PlanApo 40x oil immersion objective lens (Zeiss) with a numerical aperture value of 1.4. TMRE was excited at 555nm and emission was recorded with a band-pass

filter of 575–640nm. Measurements were taken from individual experiments and intensities were quantified relative to background levels.

4.2.6 Statistical analysis

Values are expressed as the mean ± SEM. Statistical significances between groups were determined by the use of Student's *t* test and one way ANOVA followed by Duncan's tests to assess multiple group comparisons. Values were considered significant if $p < 0.05$.

4.3 Results

4.3.1 Cardioprotective Effects of UA-8

To demonstrate whether UA-8, a structural analog of EET with sEH inhibitor properties (Fig.4-1A), has similar cardioprotective effects as EETs, we first performed a concentration-response study. We perfused C57BL/6 mouse hearts with 0, 0.01, 0.1, 0.5 and 1μM of UA-8 and monitored LVDP for postischemic functional recovery (Table.4-1). Hearts perfused with UA-8 had significantly improved postischemic recovery of LVDP compared to control mice (Fig. 4-2A). The improved postischemic recovery followed a rapid concentration-response with the improved functional recovery occurring at low concentrations (0.1μM 80.0±4.0%, 0.5μM 70.0±9.0%, 1μM 71.0±11.0%) compared to vehicle treated hearts (34.0±4.0%). The improved postischemic functional recovery from UA-8 (0.1μM) was significantly higher compared to functional recovery from sEH null mice (49.0±5.0%) or hearts perfused with 11,12-EET (1μM) (60.0±7.0) (Fig. 4-2B). Infarct size was assessed after 20 min of global ischemia and following 2 h reperfusion with UA-8(0.1μM) - injury was measured as a percentage of the infarct region to the area at risk (IS/AAR). A significant decrease in infarct size was observed in the hearts treated with UA-8 compared to both vehicle-treated and EET-treated hearts (Fig. 4-4A). Together these data suggest that UA-8(0.1μM) is a potent and stable agent and improves postischemic functional recovery similar to that of natural EETs at nanomolar concentrations.

4.3.2 Protective Effects of UA-8 is Due to EET Structural Mimicking Activity

To determine whether the improved ventricular recovery of UA-8(0.1μM) was due to EET structural properties or sEH inhibitor activity, we perfused hearts with UA-7, a synthetic compound that is structurally similar to UA-8 but possesses no sEH inhibitor properties (Fig. 4-1A). Hearts perfused with UA-7(1μM) had significantly improved postischemic recovery of LVDP compared to control mice, similar to the improved recovery seen with UA-8 (UA-7 70.0±8.0%; UA-8 71.0±11.0%; vehicle 34.0±4.0%). Next, hearts were co-perfused with the putative pan-EET antagonist 14, 15-EEZE and either UA-8 or UA-7. Interestingly perfusion of this antagonist completely abolished both the UA-8 and UA-7 improved post-ischemic functional recovery (UA-8- 24.0±7.0%; UA-7- 27.0±4.0%) (Fig.4- 3A & 4-3B). Finally, hearts were co-perfused with UA-8 and the CYP epoxygenase inhibitor MS-PPOH (50μM) (Fig.4-3A), to block the endogenous EET production. However, the administration of this inhibitor did not have any effect on the UA-8 postischemic recovery. Taken together, these data suggest that UA-8 mediated protection results from EET structural mimicking activity rather than sEH inhibitor activity.

4.3.3 PI3K Mediated Effects of UA-8

To further investigate the role of PI3K in UA-8 mediated protection, we assessed the expression levels of phosphorylated Akt and GSK3ß, immediate downstream targets.[33] Consistent with a role for PI3K, perfusion with UA-8 resulted in an increased expression of phosphorylated Akt and GSK3ß in the hearts following IR compared to vehicle controls (Fig. 4-5A & 4-5B). These data

suggest the involvement of the PI3K cascade in the cardioprotective effects of UA-8. Next, we performed isolated heart experiments with the PI3K non-selective inhibitor, wortmannin and the class-I PI3K selective inhibitor, PI-103(1μM), to confirm the role of PI3K signalling. Perfusion with wortmannin significantly reduced the improved postischemic functional recovery in UA-8 treated hearts. Interestingly, perfusion of PI-103 also abolished the improved postischemic functional recovery (Fig. 4-6A), demonstrating that class-I PI3K is involved in the UA-8 mediated cardioprotection.

4.3.4 Mitochondrial Membrane Potential ($\Delta\psi_m$)

To examine whether UA-8 affords mitochondrial protection, we conducted experiments using H9c2 cells and subjected them to 6 h anoxia followed by 16 h of reoxygenation. A marked reduction in cell death was observed following anoxia-reoxygenation in cells treated with UA-8 compared to vehicle controls (Fig. 4-7A). Consistent with these data, cells treated with UA-8 had a lower activation of caspase-3, suggesting a reduction in anoxia-reoxygenation induced apoptosis (Fig. 4-7B). Anoxia-reoxygenation resulted in the rapid dispersion of TMRE fluorescence from mitochondria (Fig. 8A), indicating dissipation of membrane potential ($\Delta\Psi_m$) and suggesting loss of mitochondrial function. Co-treatment of cells with UA-8 significantly slowed the loss of $\Delta\Psi_{m,}$ similar to an 11,12-EET treated group. Together these data imply that UA-8 can preserve mitochondrial function caused by anoxia-reoxygenation, thereby limiting cell loss. Interestingly, when cells treated with UA-8 were co-incubated with PI-103 the protective effect was abolished (Fig.4-8A). Consistent with our functional

recovery data, these results suggest that UA-8 regulates mitochondrial function in a manner similar to the EETs, and moreover, involves a class-I PI3K-mediated pathway.

4.4 Discussion

In this chapter, we present data demonstrating that UA-8, a novel dual-function compound possessing both EET mimetic and sEH inhibitory properties, improves postischemic ventricular contractile function and reduces infarct size in isolated mouse hearts. The observed cardioprotective effects were due to structural properties that mimicked EET function. Moreover, our data demonstrate that UA-8 protective mechanism(s) are mediated through a class-I PI3K-dependent pathway limiting mitochondrial damage.

EETs are chemically and metabolically labile compounds where rapid metabolism, low solubility and storage issues limit their use as therapeutic agents and pharmacological tools.[13] Exogenously applied EETs have a rapid cellular uptake and metabolism resulting in a half-life ranging from a few seconds to minutes.[18, 31] Difficulty arises with long-term treatment using EETs as they are rapidly metabolized or incorporated into membranes. [10, 11, 21] As such, considerable interest has arisen in developing methods to enhance the bioavailability of EETs. Decreasing the hydrolysis of EETs through inhibition of sEH using various urea, amide or carbamate based pharmacological agents as an approach to elevate EET levels is comparable to genetic models.[1,6,16, 23, 36] Compounds such as 1,3disubstituted urea-based old generation CDU (1-Cyclohexyl-3-dodecyl-urea), AUDA (12-(3-adamantan-1-yl-ureido)dodecanoic acid), AUDA-BE (butyl ester) and new generation inhibitors like t-AUCB(*trans*-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid) with improved oral bioavailability and stability are known to be potent inhibitors of sEH. [6,16,23]

Recent studies have confirmed the beneficial effects of sEH inhibitors against IR injury, hypertension and stroke.[4,26, 27] However, the drawback to these compounds as therapeutic agents includes rapid metabolism, limited oral bioavailability and low water solubility.[16] In addition, while these compounds succeed in inhibiting sEH and increasing EET levels, they do not prevent EET degradation by other routes such as ß-oxidation, autooxidation and esterification, and may increase EET oxidation.[8]

Structure activity relationship studies have demonstrated that EETs require a negatively charged group at the C-1 or need to be partially saturated with a double bond between 8, 9Δ-carbon to protect against autooxidation, as well as an epoxy group to possess EET functional effects.[8,9,14, 37] Replacing the epoxy group with bioisosteres like urea, thiourea and oxiamide and partially saturating the molecule increases stability and maintains functional activity while protecting against autooxidation and sEH hydrolysis.[8] UA-8 is a structural analog of EET that is partially saturated, making it less prone to oxidation, and has a 1,3-disubstituted urea replacing the epoxy group.[8] Recently, Falck *et al*. demonstrated that UA-8 mimics the vasodilator properties of EETs at much lower concentrations (100-fold) and exhibits resistance to oxidation in preconstricted bovine arteries.[8] In addition, UA-8 can inhibit sEH at nanomolar concentrations (IC_{50} 46nM) similar to that of urea based sEH inhibitors. [8] While UA-8 can potentially undergo ß-oxidation, an increased resistance to autooxidation, esterification and degradation provides a significant advantage compared to other sEH inhibitors leading to a longer duration of its protective action. In the present

study, we demonstrate that hearts perfused with UA-8 at concentrations as low as 100nm show significant improvement in LVDP preventing the loss in cardiac contractile function, similar to the cardioprotection elicited by EETs (1μM) or sEH null mice. Moreover, UA-8 significantly reduced the amount of irreversible injury, or infarction, observed in control and EET-treated hearts. Thus, demonstrating that UA-8 has better potency compared to EETs alone, most likely reflects increased stability. Improved recovery following perfusion with UA-7, which has a sEH IC_{50} of 58712 nM, suggested that the protective effect of UA-8 was attributed to EET mimetic properties as opposed to sEH inhibition. [8] This finding was further supported when UA-8 and UA-7 improved postischemic recovery was attenuated by the EET-antagonist, 14, 15-EEZE, in parallel with no effect of MSPPOH. Thus these data suggest that EET structural and functional properties are important to limiting ischemia-reperfusion injury.

Previous studies have shown EET-mediated cardioprotection involves activation of a PI3K-mediated pathway phosphorylating downstream targets like Akt and GSK3ß. [5, 7, 19, 29]PI3Ks are members of a family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphoinositides. PI3K phosphorylates phosphoinositol-4,5-bisphosphate (PIP_2) producing phosphoinositol-3,4,5-triphosphate (PIP_3), considered important lipid messenger molecules that regulate the localization and function of multiple proteins and ion channels.[34] Enhanced activation of PI3Ks and signalling pathways involving downstream kinases in IR is known to reduce cell death and infarct size.[24]Consistent with reports of EET-mediated cardioprotection, hearts perfused with UA-8 had increased expression of

activated Akt and GSK3ß. [5,7,29] Thus, the current results suggest that UA-8 reproduces EETs protective mechanisms and support the involvement of PI3K survival signalling. PI3Ks are classified into various classes (class-I, -II and -III) based on mode of activation and structure. PI3Ks from all classes produce the same secondary messenger but are known to have different mechanisms of action.[25] PI3K studies often use non-specific inhibitors, like wortmannin, making it difficult to differentiate which isoform is responsible for the protective effect. Our data demonstrate that the class-I specific PI3K inhibitor, PI-103, abolished the UA-8 protective action, suggesting a role for these kinases in the protective response.

Mitochondria are strategic regulators of life and death, playing a central role in energy production, calcium homeostasis and programmed cell death. Ischemic injury causes distinct morphological changes to mitochondria impacting dynamics and function.[3] Activation of PI3K initiates survival pathways that can prevent the collapse of mitochondrial function following injury. [17, 35] Mitochondrial membrane potential ($\Delta\Psi_m$), a marker of mitochondrial activity, can provide information about cellular function following significant cell stress conditions and loss of $\Delta\Psi_m$ ultimately contributes to contractile dysfunction and cell death. [17] In the present study, incubation of H9c2 cells with UA-8 slowed the loss of $\Delta\Psi_m$ following anoxia-reoxygenation injury. Importantly, the effects of UA-8 were blocked by co-administration of PI-103, suggesting a role for class-I PI3K isozymes in maintaining mitochondrial function following cellular injury. In

addition, UA-8 treatment reduced activation of caspase-3 activity limiting the apoptotic cell death attributed to an IR injury.

In summary, we report improved postischemic contractile function and reduced infarct size with the dual-action compound, UA-8, possessing EET mimetic and sEH inhibitory properties. Moreover, our data suggest that the EET mimetic properties are important for the improved recovery and class-I PI3K are involved in the protective mechanism. The increased potency and stability of UA-8 may serve as a potential therapeutic agent in limiting mitochondrial damage and myocardial injury attributed to ischemia/reperfusion events.

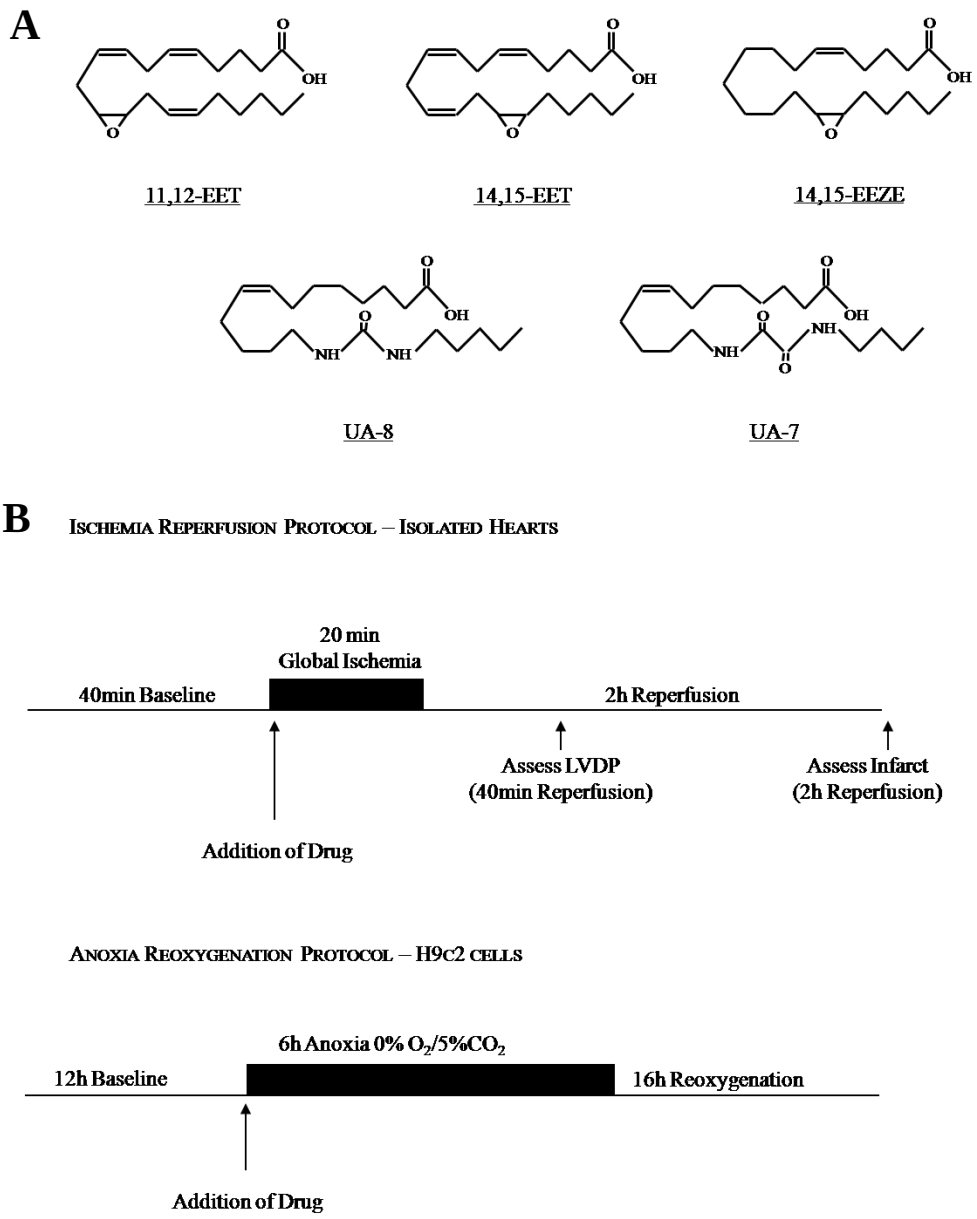

Fig. 4.1 (A) Structures of 11, 12-EET, 14, 15-EET, 14, 15-EEZE, UA-7 and UA-8. (B) Experimental protocols for ischemia/reperfusion and anoxia/reoxygenation. LVDP, left ventricular developed pressure.

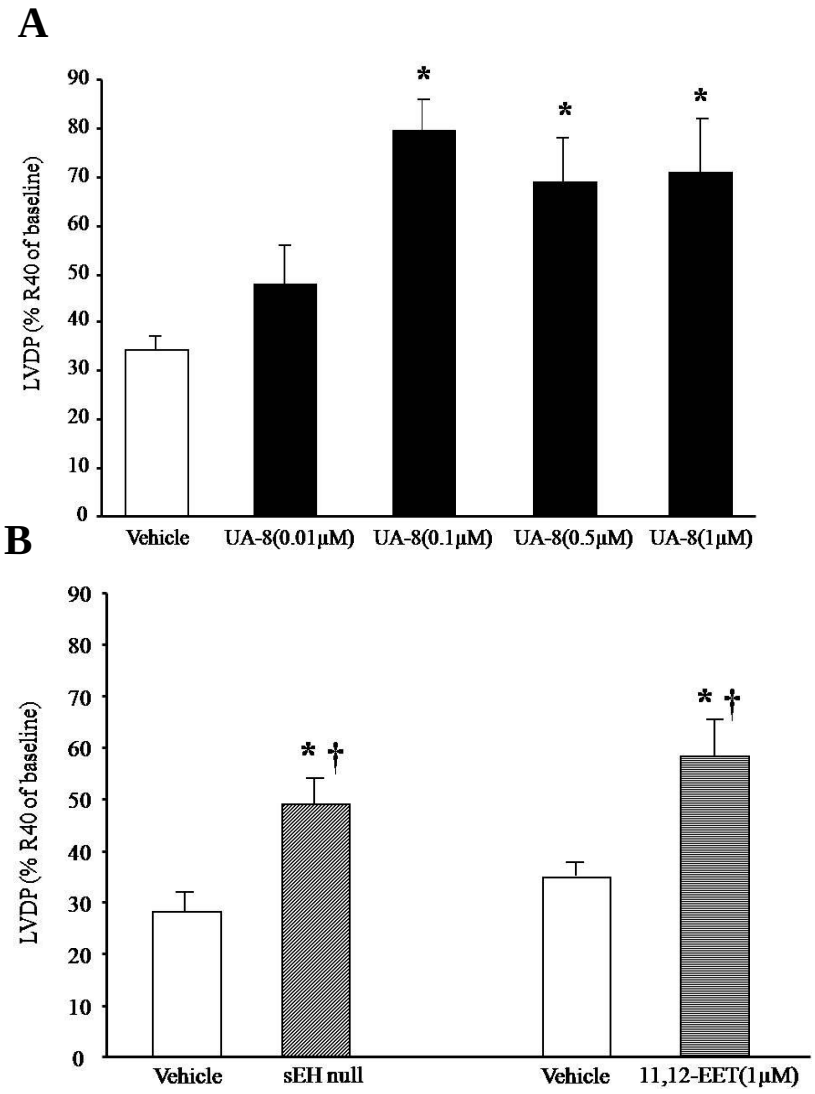

Fig. 4.2 (A) Histogram of the postischemic functional recovery of LVDP at 40 min of reperfusion, expressed as percentage of baseline LVDP from hearts treated with vehicle or UA-8 (0.01, 0.1, 0.5 and 1 μM). Values represent means ± SEM, *n* = 5–11 per group; **P* < 0.05 versus vehicle control. (B) Histogram of the postischemic functional recovery of LVDP at 40 min reperfusion expressed as percentage of baseline LVDP from sEH null and WT mice treated with vehicle or 11, 12-EET (1μM). Values represent means ± SEM, *n* = 7–11 per group; **P* < 0.05 versus vehicle control.

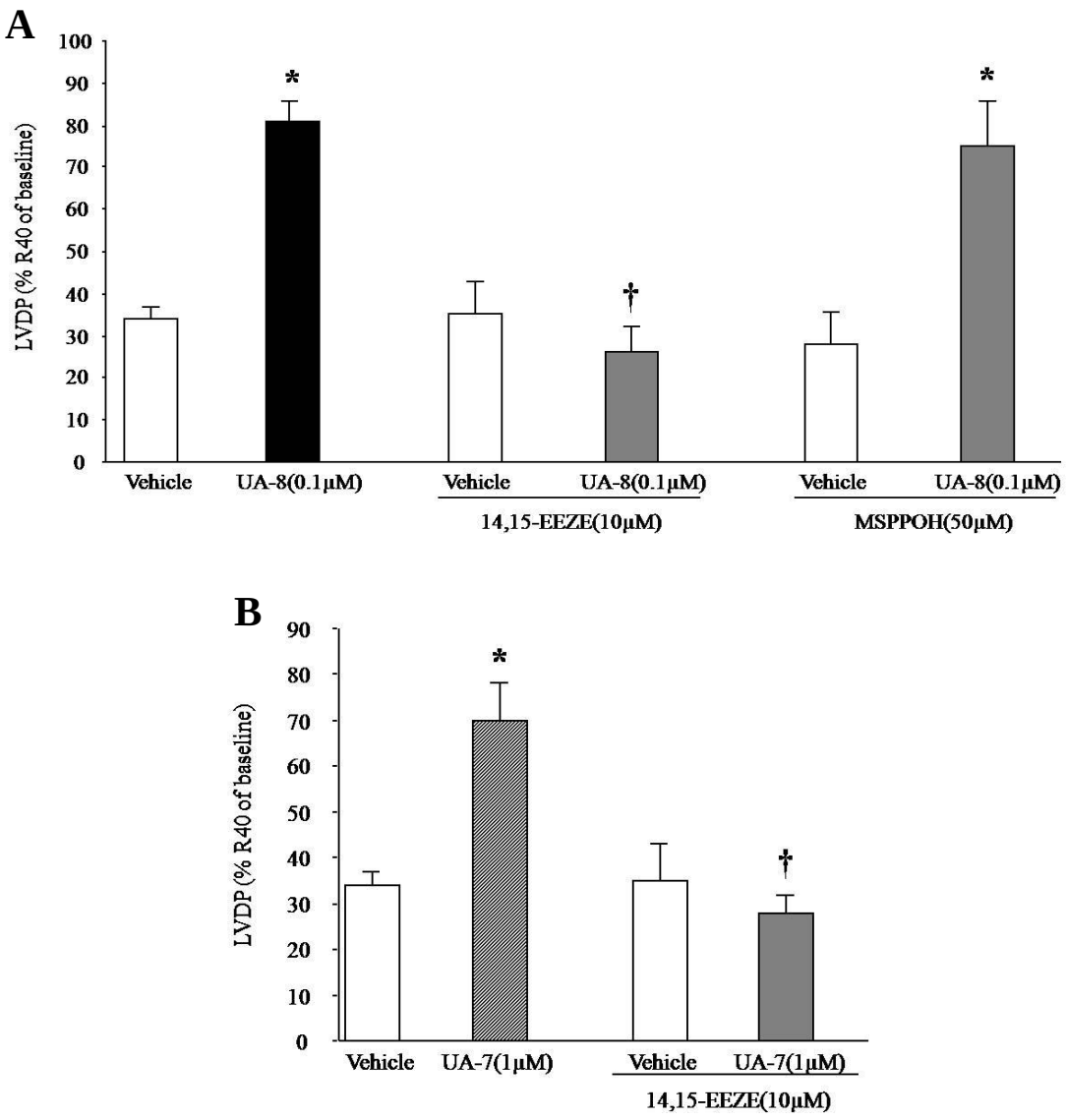

Fig. 4.3 (A) Histogram of the functional recovery at 40 min reperfusion expressed as percentage of baseline LVDP from hearts perfused with vehicle or UA-8 (0.1 μM), 14,15-EEZE (10μM) or MS-PPOH (50 μM) following 20 min of ischemia; *n* = 3–11 per group. (B) Histogram of the functional recovery at 40 min reperfusion from hearts perfused with vehicle or UA-7 (1μM) and 14,15-EEZE (10μM) following 20 min of ischemia; *n* = 3–4 per group.

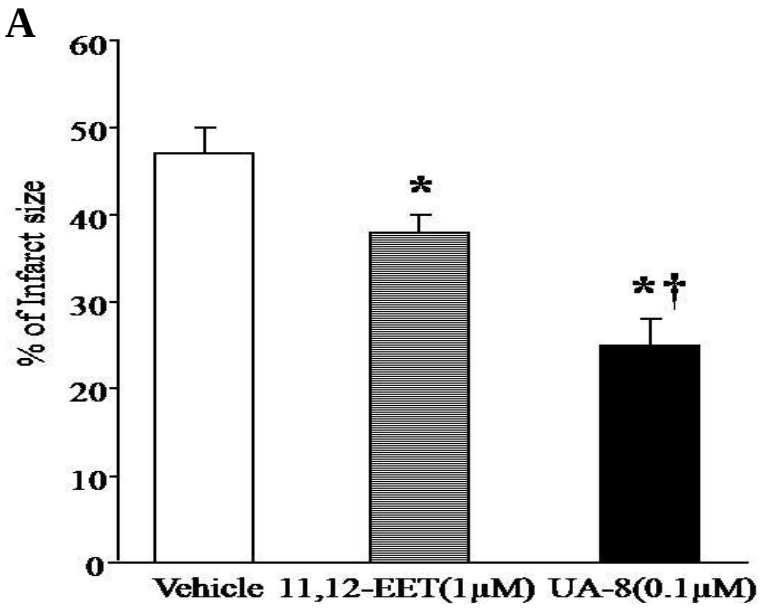

Fig. 4.4 (A) Quantification of infarct size from the hearts perfused with vehicle, 11,12-EET (1μM) and UA-8 (0.1μM) following 20 min of ischemia; n = 3–4 per group. Values represent mean ± SEM, *$P < 0.05$ versus vehicle control; †$P < 0.05$ versus drug treated group.

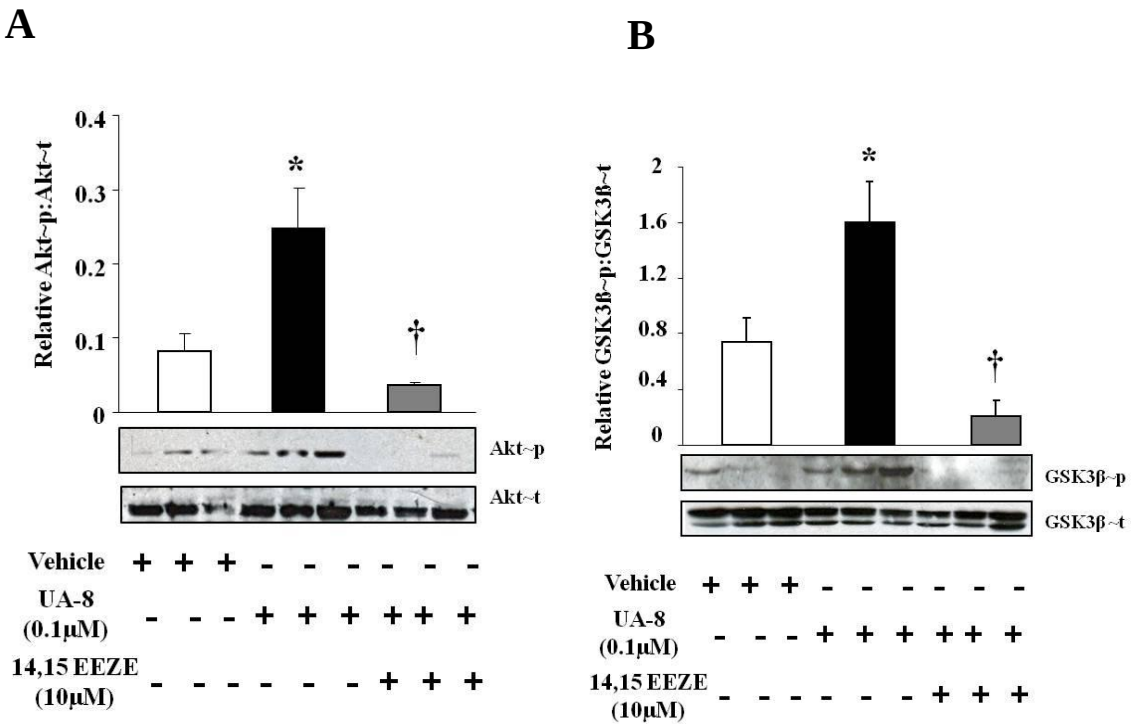

Fig. 4.5 (A) Immunoblot and densitometry showing the ratio of Akt-p to Akt-t expression in C57BL/6 hearts treated with vehicle, UA-8 (0.1μM) or 14,15-EEZE (10μM). (B) Immunoblot and densitometry showing the ratio of GSK3β-p to GSK3β-t expression in C57BL/6 hearts treated with vehicle, UA-8 (0.1μM) or 14, 15-EEZE (10μM). Values represent mean ± SEM, n = 3 per group; *P < 0.05 versus vehicle control; †P < 0.05 versus treated group.

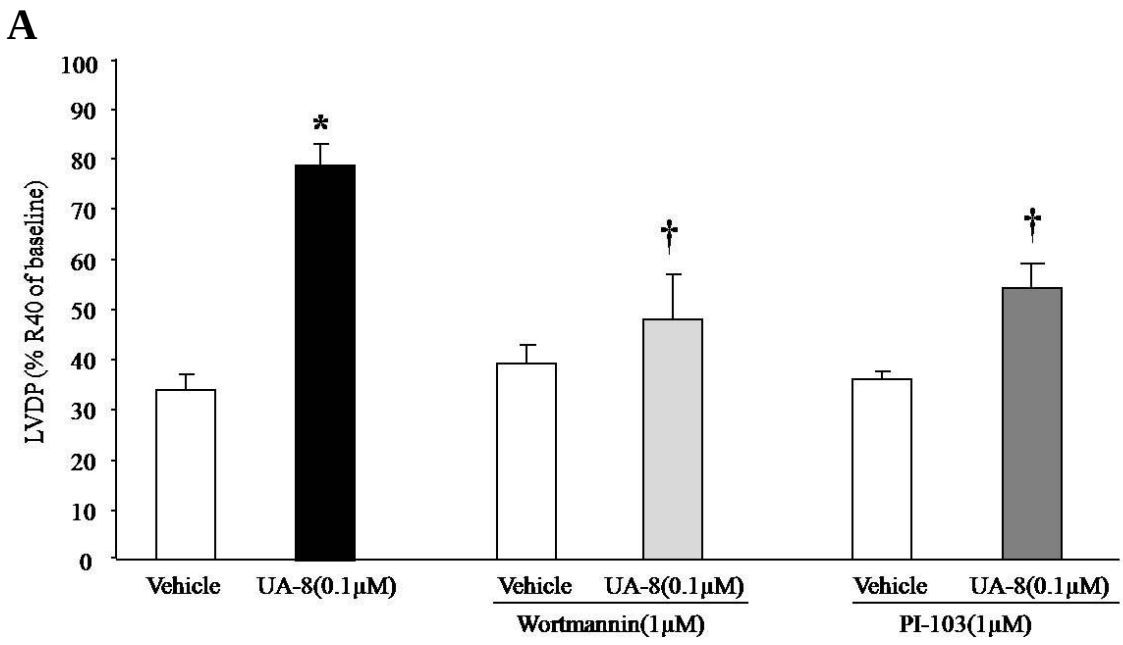

Fig. 4.6 (A) Histogram of the percentage of left ventricular developed pressure (LVDP) change at 40 min reperfusion compared to baseline from the hearts perfused with vehicle or UA-8 (0.1μM), wortmannin (1μM) and PI-103 (1μM) following 20 min of ischemia. Values represent means ± SEM, n = 3–6 per group; *$P < 0.05$ versus vehicle, †$P < 0.05$ versus UA-8-treated group.

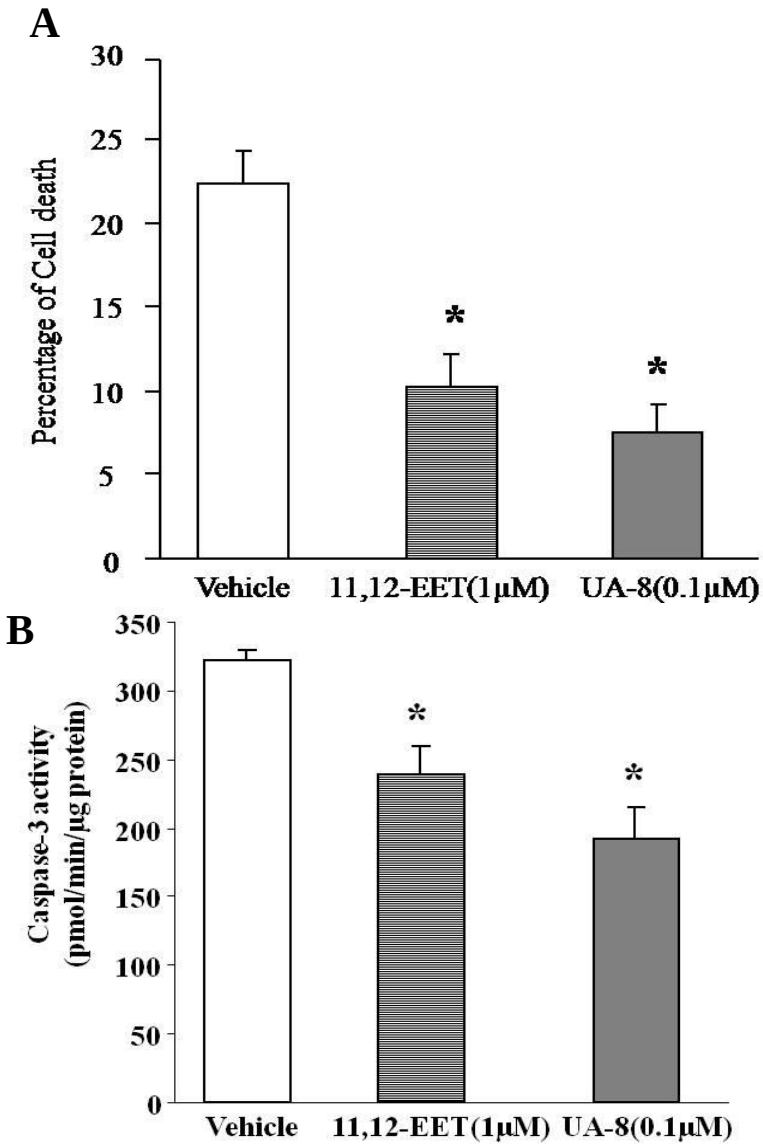

Fig. 4.7 UA-8 limits loss of mitochondria function. (A) Cell viability: histogram representing the percentage of cells that died after being subjected to 6 h of anoxia and 16 h reoxygenation and treated with vehicle, 11,12-EET (1 μM) or UA-8 (0.1 μM). Values represent mean ± SEM; n = 4–5; *P < 0.05 versus vehicle control. (B) Caspase-3 activity in H9c2 cells using Ac-DEVD-AMC as substrate. Cells subjected to 6 h of anoxia and 16 h of reoxygenation were treated with vehicle, 11,12-EET (1 μM) or UA-8 (0.1 μM). Values represent mean ± SEM; n = 4–5; *P < 0.05 versus vehicle control.

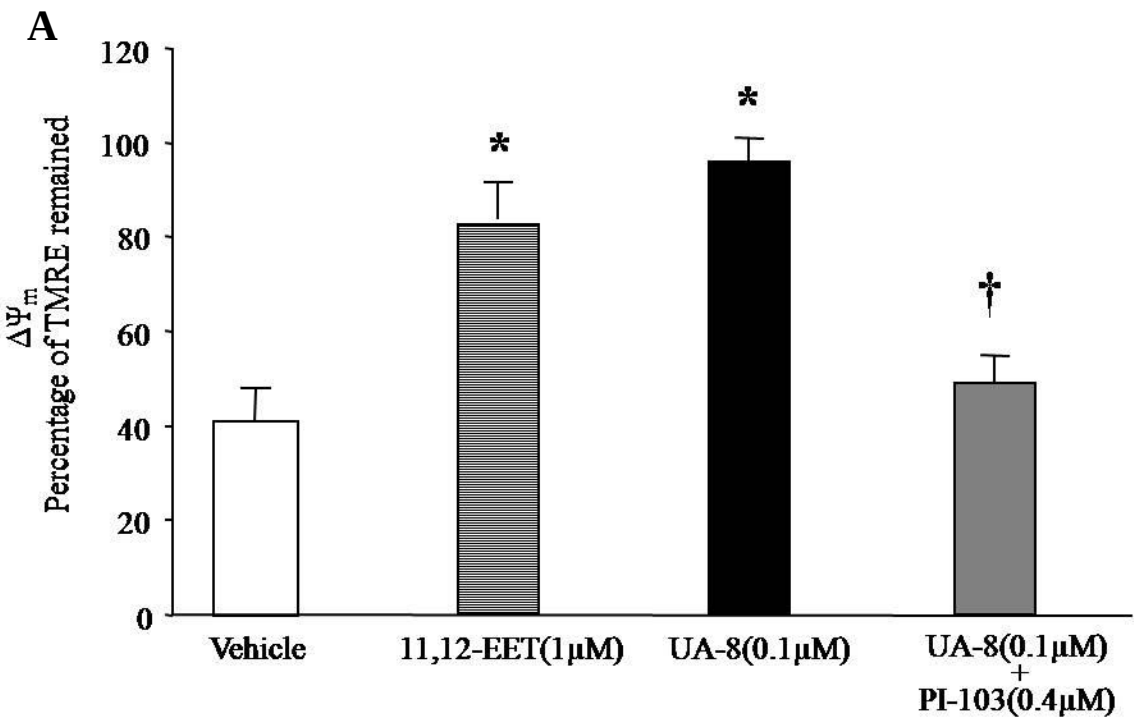

Fig. 4.8 (A) Mitochondrial membrane potential (ΔΨm). Histograms representing the percentage of tetramethylrhodamine ethyl ester (TMRE) fluorescence lost in H9c2 cells following collapse of ΔΨm. Cells subjected to 6 h of anoxia and 16 h of reoxygenation were treated with vehicle, 11,12-EETs (1 μM), UA-8 (0.1 μM) or PI-103 (0.4 μM). Values indicate % change in relative fluorescence from baseline, values represent mean ±SEM; n = 4–5, *P < 0.05 versus vehicle control; †P < 0.05 versus treated group.

	Vehicle control (*n*=11)	UA-8 (0.01μM) (*n*=4)	UA-8 (0.1μM) (*n*=6)	UA-8 (0.5μM) (*n*=4)	UA-8 (1μM) (*n*=5)
Isolated Perfused Heart – Preischemic					
LVDP (cmH_2O)	114.0±7.0	122.0±11.0	100.0±4.0	122.0±2.0	108.0±7.6
Rate of contraction, dP/dt_{max} (cmH_2O /msec)	3856±485	4235±258	3628±371	5428±1002	3056±477
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O /msec)	-2885±370	-3322±237	-3244±291	-4527±737	-2613±413
HR, perfused (beats/min)	330±21	281±57	328±23	318±16	289±34
***Isolated Perfused Heart - Postischemic*(R40)**					
LVDP (mmHg)	39.0±4	58.0±5	80.0±7*	85±16*	77±13*
Rate of contraction, dP/dt_{max} (cmH_2O /msec)	1326±209	1600±147	2588±404*†	2963±506*†	2336±344*†
Rate of relaxation, $-dP/dt_{min}$ (cmH_2O /msec)	-1108±179	-1737±282	-2785±347*†	-2712±455*	-2273±375*†
HR, perfused (beats/min)	303±18	279±18	336±16	297±12	237±40

Table 4.1 Hemodynamic parameters measured in isolated-perfused hearts following perfusion with different doses of UA-8. Values represent mean±SEM, * $P<0.05$ vs Vehicle control, †$P<0.05$ vs UA-8(0.01μM).

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CHAPTER-V

Role of PI3Kα and Plasmamembrane ATP-Sensitive Potassium Channels in Epoxyeicosatrienoic Acid Mediated Cardioprotection[5]

The following individual participated in experiments used to collect the data for this chapter.

Dr. Wei Y conducted the electrophysiology experiments

[5]*A version of this chapter has been submitted to Cardiovascular Research Journal: Batchu SN, El-Sikhry H, Chaudhary KR, Wei Y, Light PE, Oudit GY, Seubert JM. Role of PI3Kα and Plasmamebrane ATP-Sensitive Potassium Channels in Epoxyeicosatrienoic Acid Mediated Cardioprotection.*

5.1 Introduction

In recent years, it has become evident that metabolites of polyunsaturated fatty acids act as critical intracellular mediators, maintaining cardiac homeostasis and initiating protective responses to cellular stress. Arachidonic acid (AA) is a polyunsaturated fatty acid normally found esterified to cell membranes that can be released in response to several stimuli including ischemia.[1] Free AA is further metabolised by cyclooxygenases, lipoxygenases, and cytochrome P450 (CYP) epoxygenases to numerous products collectively termed eicosanoids with differing cellular function in health and disease.[2, 3] CYP epoxygenases metabolize AA to four regioisomers (5,6-, 8,9-, 11,12- and 14,15) products, epoxyeicosatrienoic acids (EETs).[2, 3] Elevated levels of EETs are known to render cardiac and extra-cardiac protection against injury.[4-6] While the exact mechanism remains unknown, evidence indicates EETs protect the heart by modulating ion channels such as *pm*K_{ATP} and activating PI3K pathways, thereby effecting cardiovascular physiology and function.[6-10]

Cardiac *pm*K_{ATP} channels are involved in regulating ionic homeostasis under conditions of metabolic stress and have demonstrated cardioprotective effects towards ischemia reperfusion injury.[11, 12] *pm*K_{ATP} channels can be activated during cardiac ischemia when cytoplasmic ATP is depleted and affects membrane excitability. Activation of *pm*K_{ATP} channels during ischemia leads to shortening of the cardiac action potential and opposes membrane depolarization[13, 14] consequently reducing intracellular calcium overload thus limiting myocardial damage.[14, 15] Recent data from animal

models of IR injury have demonstrated that EET-mediated activation of *pm*K_{ATP} channels improves cardiac functional recovery.[4, 6, 9, 10] While EETs have been shown to be activators of *pm*K_{ATP} by reducing channel sensitivity to ATP under normoxic conditions[10], the mechanism(s) of how this occurs during IR injury remains enigmatic.

PI3Ks are members of a family of lipid kinases that regulate a range of cell survival, growth and metabolic processes.[16] Based on amino acid sequence, structure and mode of activation, these are divided into three classes (I, II and III).[17] Class I PI3K catalyze the addition of a phosphate group to 3'position of the phosphatidylinositol (PIP),[16, 17] activating other intracellular signalling kinases [16, 17] and ion channels [18] with distinct roles in regulating cardiac function.[19-21] Enhanced activation of class 1 PI3Ks and signalling pathways involving downstream kinases, such as AKT and GSK-3β, during IR injury results in reduced cell death and infarct size.[17] PI3K pathways have been demonstrated to be involved in EET cardioprotective signalling.[6, 8]

While EET-mediated cardioprotection involves PI3K and *pm*K_{ATP} channel dependent events, the specific intracellular signaling mechanism(s) remains unresolved. In this study, we identify the specific PI3K isoform (PI3Kα) involved in the EET protective mechanism and investigate the link between PI3K and *pm*K_{ATP} channels in EET cardioprotection. Furthermore, our results suggest that activation of PI3Kα and *pm*K_{ATP} channels by EETs renders protection by decreasing Ca^{2+} overload following ischemic injury, limiting mitochondrial damage.

5.2 Materials and methods

5.2.1 Animals

Commercially available C57BL/6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ). The PI3Kγ-/- [21] and Kir6.2KO [12] (K_{ATP} channel-deficient) mice were used as previously described. All studies were carried out using mice aged 3 month and 25-30g. Experiments were conducted according to strict guidelines provided by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS). The investigation conforms to 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).

5.2.2 Isolated Heart Perfusion

Hearts were perfused in the Langendorff mode as described previously.[7, 22] Mice were euthanized intraperitoneally with sodium pentobarbital (Euthanyl (500mg/kg)) in a terminal procedure anaesthetized intraperitoneally with pentobarbital. Once the mouse was nonresponsive to consecutive foot pinches with tweezers, an incision was made longitudinally through the skin and muscle to expose the abdominal cavity. Following this heparin was administered intravenously through the dorsal vein to prevent blood coagulation. An incision was made from the diaphragm to the throat to cut open the thoracic cage so as to expose the heart. Following this the heart was separated from the body using blunt dissection. The isolated heart was mounted to a 23-gauge needle attached to the perfusion apparatus through the aorta. Then heart was perfused in a retrograde fashion at constant pressure (80cmH_2O) in the Langendorff mode with

continuously aerated (95%O_2/5%CO_2) Krebs-Henseleit buffer(NaCl 120mM, glucose 10mM, $NaHCO_3$ 25mM, $CaCl_2$ 1.75mM, $MgSO_4$ 1.20mM, KH_2PO_4 1.20mM, KCl 4.70mM) maintained at 37^oC. For assessment of the left ventricular functional parameters (LVDP), an incision was made in the left atrium and a saline filled balloon connected to a transducer was inserted through the incision into the left ventricle. Chart software (ADInstruments) was used to record the functional data. Ischemia reperfusion experiments, hearts were perfused with buffer for 40min to stabilization them, then subjected to 20 or 30min global no-flow ischemia followed by 40min reperfusion (Fig. 1A). In some experiments hearts were perfused with 11,12-EET and/or PI-103(PI3Kα inhibitor, Cayman Chemicals, USA), TGX-221 (PI3Kβ inhibitor, Cayman Chemicals, USA) or wortmannin (pan-specific inhibitor, Sigma-Aldrich, Canada) and were added 5min before ischemia and present in the heart during ischemia and throughout the time of reperfusion. Recovery of contractile function (LVDP) at the end of reperfusion was expressed as a percentage of preischemic LVDP. At the end of the experimental protocol heart tissues were snap frozen and were kept in -80^oC until needed for further experiments.

5.2.3 ***Isolation of fresh adult cardiac ventricular myocytes***

Myocytes were isolated from mice enzymatically. Briefly, mice were intraperitoneally heparinized (0.1ml heparin (5000U/Kg IP)) and then anesthetized with pentobarbital sodium (50 mg/kg). Hearts were quickly removed and cannulated the aorta on to a Langendorff apparatus. Followed by perfusion at a rate of 2-3ml/min with Ca^{2+} free with a solution containing NaCl 120mM, KCl

5.4mM, $MgSO_4$ 1.20mM, KH_2PO_4 1.20mM, $NaHCO_3$ 20mM, glucose 5.6mM, BDM 500mM , Taurine 5mM, HEPEs 1M, pH 7.4. Subsequently, the perfusion buffer was replaced with the same buffer containing collagenase B (Roche, Canada), collagenaseD (Roche, Canada), protease (Type XIV, Sigma-Aldrich, Canada). All the solutions were maintained at 37 °C temperature. Following 6-8 min of perfusion with this enzyme solution, the heart was cut to small pieces, minced and pipette gently using plastic transfer pipettes for couple of minutes. Then the cell suspension was transferred to 12ml sterile culture tube and the volume was made up to 5ml with buffer containing Ca^{2+} free solution, fetal bovine serum and $CaCl_2$ (50μM) and continue to pipette up and down using different diameter plastic transfer pipettes until all the large chuncks are dispersed. This cell suspension was filtered through 100μM nylon cell strainer in to 50ml Falcon tube following this filtrate was transferred to 4ml sterile cell culture tubes and allowed the myocytes to settle by gravity for 8-10min. Following this the supernatant was removed and to this another buffer containing Ca^{2+} free solution, fetal bovine serum and $CaCl_2$ (200μM) and allowed the myocytes to settle by gravity. Again following this supernatant was removed and replaced with buffer solution containing Ca^{2+} free solution, fetal bovine serum and $CaCl_2$ (500μM). Myocytes with clear striation patterns were used for the electrophysiological studies.

5.2.4 *Electrophysiology*

Whole-cell K_{ATP} channel currents were recorded from freshly isolated mouse ventricular myocytes that were placed in a recording chamber on the stage

of an inverted microscope and superfused with an extracellular bath solution containing (mM) NaCl 20, KCl 4.5, choline chloride 130, $CaCl_2$ 1, $CoCl_2$ 2, $MgCl_2$ 2, HEPEs 10, and glucose 5.5, pH 7.4. After gigaohm seal formation, K_{ATP} channel currents were elicited by rupturing the patch and dialyzing the cells with a pipette solution containing (mM): KCl 140, $CaCl_2$ 0.465 (200nM free Ca^{2+}), $MgCl_2$ 0.5, Na_2ATP 1, Na_2GTP 0.5, HEPES 1, EGTA 1, pH 7.3. Currents were filtered at 2 kHz and digitized at 5 kHz using an Axopatch 200B amplifier and pClamp 8.2 software (Axon Instruments, Union City, CA, USA). Experiments were performed at a negative holding potential of –100mV at room temperature (21–23°C). 10μM glibenclamide (Glib, a K_{ATP} channel inhibitor) was used to confirm the identity of the the K_{ATP} channel current. 11, 12 EET was applied to the myocytes in the bath solution. In some experiments, myocytes were pretreated with the PI3Kα inhibitor PI-103 (0.1μM) for 30 min prior to current recording and PI-103 was also present in the bath solution throughout the experiment. Pipette resistance was 1–3 MΩ when filled with the pipette solution. K_{ATP} channel current data are expressed in normalized form i.e. test current divided by the steady state current before 11, 12 EET application.

5.2.5 Cell culture experiments

H9c2 cells (American Type Culture Collections, Manassas, VA) were cultured in 75cm^2 flasks at 37°C in an atmosphere of 5% CO_2/ 95% air. DMEM media with phenol red, supplemented with 10% fetal bovine serum, and antibiotics such as penicillin, streptomycin and amikacin, were used to nourish the cells but in some experiments we used Ca^{2+} free DMEM media. In all

experiments, cells were seeded on petri dishes and were treated with either 11, 12-EET, PI-103 or glibenclamide (GLIB) (Fig. 1B). Cell viability was assessed by using trypan blue dye exclusion assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) assay. For the trypan blue method following anoxia–reoxygenation cells were collected along the media and briefly centrifuged. The resultant supernatant was discarded and the pellet was resuspended in homogenization buffer. A 50 µl aliquot was mixed with 0.4% trypan blue dye (Sigma-Aldrich, Oakville, ON) in a 1:1 ratio and this mixture was then placed on the hemocytometer and viable (unstained) and dead (blue-stained) cells were counted. For the MTT assay, after experimental treatment 20µl (5 mg/ml) of MTT was added to each well and incubated for 2hr. Following this the medium was removed and 200µl of DMSO was added to each well to dissolve the formazan crystals formed by oxidation of the MTT dye. The absorbance of the blue formazan derivative was measured at 490nm and the cell survival ratio was expressed as a percentage of the control.

5.2.6 Measurement of reactive oxygen species

Reactive oxygen species (ROS) levels were measured by using 2.5µM DCF dye (Invitrogen, USA) in Hank's buffer following anoxia-reoxygenation.

5.2.7 Calcium overloading experiments

Ca^{2+} overloading experiments were done by subjecting the H9c2 cell to anoxia and reoxygenation. Anoxic conditions were generated by replacing the media with de-oxygenated Hank's buffer. Following this, the glass-bottom petri dishes with cells were mounted on an epiflourescence microscope stage (Carl

Zeiss) and for 1 hour anoxic conditions were maintained by flushing 5% CO_2/ 95% N_2 gas. Reoxygenation was obtained after the stipulated anoxic period by adding oxygenated Hank's buffer maintained at 37^oC and by flushing with 5% CO_2/ 95% air. Ca^{2+} levels were measured by adding Fluo-4(2µM) dye to the oxygenated hanks buffer. The dye was excited at 470nm using LED at 5% of maximum intensity and emission was recorded between 500-550nm. Cells were scanned every 10sec for 60sec. Relative Ca^{2+} fluorescence was measured using AxioVision software and data were presented in arbitrary fluorescence units.

5.2.8 Mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was studied under oxidative stress conditions maintained for 6h followed by 16h of reoxygenation. Anoxic conditions, 37°C in an atmosphere of 5% CO_2/ 95% N_2, were generated in an air tight chamber (MIC101, Billups-Rothenberg, Inc.). Tetramethylrhodamine ethyl ester (TMRE) (Invitrogen, USA) was used to measure the changes in membrane potential. [10] Cells were incubated with 150nM TMRE for 20 minutes after the stipulated anoxic-reoxygenation conditions. Then they were placed under an epifluorescence microscope with a PlanApo 40x oil immersion objective lens with numerical aperture of 1.4(Carl Zeiss). TMRE-fluorescence was excited at 475nm by the LED at 5% of maximum intensity and emission was recorded at 640nm. Measurements were taken from individual experiments using AxioVision software and data were presented in arbitrary fluorescence units.

5.2.9 Calcineurin and Caspase-3 Assay

The cytosolic fraction was separated from the heart tissue as mentioned above in the western blotting and fractionation section. [4, 7] Following separation, free phosphates were removed from the cytosolic fraction by desalting the fraction using desalting columns. Before adding the cytosolic fraction the resin in the column was hydrated with phosphate-free distilled water and allowed to set overnight at 4^0C (supplied by the company–Calbiochem Calcineruin Assay Kit). After hydrating the column it was equilibrated by lysis buffer. Following this, the column was placed in a fresh centrifuge tube and cytosolic fractions, up to 350µl, are added to the column and fraction was collecting by centrifuging the columns. After desalting, the sample is stored at -80^{o}C. Phosphatase activity was measured by adding RII phosphopeptide to 5µl of sample and allowing the reaction to proceed at room temperature for 30min. Following stipulated incubation time the reaction was terminated by adding 100µl of GREEN reagent (supplied in the kit) and allowed to incubate for another 30min. The absorbance was read at A_{620} on the colorimetric plate reader. The phosphatase activity was determined from the slope and Y-intercept obtained by plotting standard curve.

Caspase-3 activity was assessed in heart cytosolic fractions using a spectrofluorometric assay as described.[22]

5.2.10 Immunoblotting

Phopshorylated and/or total proteins were measured from heart tissues and H9c2 cells using western blotting analysis as described previously[7, 22]. Hearts were homogenised in homogenisation buffer (sucrose 250mM, Tris-HCl (pH 7.4)

10mM, EDTA 1mM, Na_3VO_4 1mM, NaF 1mM) with protease inhibitors (Aprotinin 10µL/100mL, Leupeptin 2µL/100mL, Pepstatin100µL/100mL). The crude homogenate was subjected to differential centrifugation to separate the cellular fractions. Firstly the crude fraction was centrifuged at 750g for 10min. The supernatant obtained in this step was separated from the membrane fraction and further centrifuged at 10,000g for 20min. The resultant supernatant was removed to a new tube and the pellet was re-suspended in homogenisation buffer and stored as mitochondrial fraction. The supernatant was further centrifuged at 100,000g for 45min and the resultant supernatant was separated and stored at -80^{o}C as cytosolic fraction. After separation the amount of protein content in each fraction was quantified by Bradford protein assay. Following quantification proteins (75µg) were separated on12% SDS-PAGE gel and then transferred on to a nitrocellulose membrane. The blot with proteins was incubated in the 5% blocking solution (5% skim milk) for 2 hours at room temperature. Following this blots were washed with TBS-T for 15min and then incubated with Akt-t/p (1:1000), ERK 1/2-t/p (1:1000), BAD (1:500) (Cell Signalling Technology, Inc. USA) or prohibitin (1:1000) (Fitzgerald, Concord, MA, USA) antibodies overnight at 4^{o}C. After incubation, the primary antibody was removed and blots were washed again 3 times with TBS-T for 15min and were incubated with the appropriate secondary anti-body coupled with horseradish peroxide (HRP) at a dilution of 1:5000 overnight at 4^{0}C. Following incubation the blots were washed again 3 times with TBS-T for 15min and proteins were detected by enhanced chemiluminescence (GE) using X-ray films (Kodak). Relative band intensities

were expressed in arbitrary units assessed using Image J software (USA, NIH, and Bethesda, MD).

5.2.11 Statistical analysis

Values are expressed as the mean ± SEM. Statistical significance between the groups were determined by the use of the Student's *t* test and one way ANOVA followed by Duncan's tests to assess multiple group comparisons. Values were considered significant if $P<0.05$.

5.3 Results

5.3.1 *PI3Kα is responsible for EET-mediated improved postischemic functional recovery*

In previous studies we have demonstrated that PI3K is important in EET-mediated cardioprotective responses [6, 23], however, the specific PI3K isoform involved was unknown. To elucidate the isoform involved, PI3K specific inhibitors PI-103 (PI3Kα inhibitor), TGX-221 (PI3Kβ inhibitor) or PI3Kγ knockout mice (PI3Kγ -/-) were utilized with 11,12-EET. C57BL/6 hearts perfused with 11,12-EET(1μM) had significantly improved postischemic recovery of LVDP following 30min ischemia compared to vehicle treated control. Conversely, co-perfusion of PI-103 (0.01μM, 0.1μM and 1μM) with 11,12-EET (1μM) abolished the EET-improved postischemic functional recovery similar to hearts co-treated with wortmannin (200nM) and 11,12-EET (Fig. 5.1A, 5.1B, 5.4A and 5.4B). Perfusion with TGX-221 (0.01μM) reduced the postischemic functional recovery equally in both control and 11,12-EET treated hearts. Failing to block an EET-mediated effected (Fig. 5.2A and 5.5A) suggests that the PI3Kβ isoform is not involved. Perfusion with 11,12-EET to hearts from PI3Kγ-/- mice resulted in a significant increase in postischemic functional recovery similar to C57BL/6 hearts treated with EETs (Fig. 5.2 B and 5.5B), suggesting that the PI3Kγ isoform is not involved in the EET-mediated cardioprotection. Furthermore, perfusion with PI-103 to hearts from PI3Kγ-/- mice abolished the 11,12-EET-mediated improved recovery (Fig. 5.2B and 5.5B). While TGX-221 (0.01μM) failed to block the EET-mediated effects over vehicle control (Fig.

5.3A). Together, this data indicates that PI3Kα is the key isoform involved in the EET-mediated cardioprotection.

Earlier studies have suggested that the putative *mito*K_{ATP} channel was responsible for EET-mediated cardioprotective responses, in addition to *pm*K_{ATP} channels [4, 5, 10]. As the molecular composition of *mito*K_{ATP} channels remains unknown, studies have been limited to pharmacological analysis. To better address the role of *pm*K_{ATP}, hearts from Kir6.2-/- mice were perfused with vehicle or 11,12-EET (1μM) and subjected to 20min of global ischemia followed by 40min of reperfusion. Disruption of a functional K_{ATP} channel in Kir6.2-/- mice abolished the improved postischemic functional recovery attributed to 11,12-EET (Fig.5.3B and 5.6A), demonstrating that *pm*K_{ATP} channels are required for EET-mediated improved postischemic functional recovery.

Consistent with the role of PI3Kα we observed differences in the expression of p-Akt following IR injury. C57BL/6 hearts perfused with 11,12-EET (1μM) had significantly higher expression of p-Akt compared to vehicle treated hearts at 40 min of reperfusion. Inhibition of 11,12-EET improved postischemic recovery with PI-103 (0.1μM) abolished the increased expression of p-Akt at reperfusion (Fig. 5.7A). While perfusion of TGX-221 (0.01μM) had no effect on the p-Akt expression and PI3Kγ-/- mice had decreased expression of p-Akt group as shown in previous studies.[20] Interestingly, hearts from PI3Kγ-/- mice perfused with 11,12-EET (1μM) had a significant increase in p-Akt expression (Fig. 5.7A). No difference in the expression of p-Akt between the vehicle treated C57BL/6 hearts and Kir6.2-/- at reperfusion, however, when

11,12-EET (1μM) was perfused through Kir6.2-/- hearts there was significant increased expression of p-Akt (Fig. 5.7B). These data provide further evidence that PI3Kα is involved in the EET-mediated protective mechanism, moreover suggesting activation of PI3K occurs upstream of *pm*K_{ATP} channels.

To determine if PI3Kα was necessary for the 11,12 EET-mediated activation of *pm*K_{ATP} channels, *pm*K_{ATP} channel currents were measured in mouse ventricular myocytes isolated from WT mice using whole-cell patch clamp technique. Upon cell-rupture and dialysis into the cell of a pipette solution containing a reduced concentration of ATP (0.5 mM), whole-cell *pm*K_{ATP} channel currents (Fig. 5.8A, 5.8B and 5.8C) were significantly increased following application of 11,12-EET (5μM) (6.24 ± 0.17-fold increase). The 11,12-EET induced current was sensitive to inhibition by GLIB (10μM), a K_{ATP} channel inhibitor (Fig. 5.8A, 5.8B and 5.8C). Pre-treatment of myocytes with the PI3Kα inhibitor PI-103 (0.1 μM) significantly reduced the observed 11,12-EET (1μM) activation of *pm*K_{ATP} channels to 1.93 ± 0.23-fold of the pre-11,12 EET current levels (Fig. 5.8A, 5.8B and 5.8C). Taken together, these data support the notion that PI3Kα is a necessary component of 11,12-EET induced activation of *pm*K_{ATP} channels in ventricular myocytes.

5.3.2 *Intracellular effects*

Significant increases in intracellular Ca^{2+} levels caused by ischemic events can result in the activation of numerous enzymes such as calcineurin.[24] Hearts from C57BL/6 and Kir6.2-/- had increased calcineurin activity at reperfusion, perfusion with 11,12-EET (1μM) significantly decreased calcineurin activity in

C57BL/6 mice but not Kir6.2-/- mice (Fig. 5.9A). Increased intracellular Ca^{2+} can initiate cell death mechanisms triggering the movement of pro-apoptotic proteins such as BAD to mitochondria.[25] Following IR injury, we observed greater expression of BAD protein in mitochondrial fractions of control mice (Fig. 5.9B). Consistent with changes to calcineurin activities, C57BL/6 hearts perfused with 11,12-EET had reduced BAD expression and was observed in both vehicle control and 11,12-EET perfused hearts from Kir6.2-/- mice had increased mitochondrial expression of BAD (Fig. 5.10A). Consistent with initiation of apoptotic events, C57BL/6 and Kir6.2-/- hearts had increased caspase-3 activity following IR injury. Perfusion with 11,12-EET attenuated the IR induced caspase-3 activity in C57BL/6 hearts but not Kir6.2-/- (Fig. 5.10B). Together suggesting EETs protective effects involve regulating intracellular Ca^{2+} overload and mitochondrial induced apoptosis via *pm*K_{ATP} channels.

5.3.3 *Mitochondrial membrane potential ($\Delta\psi_m$) following anoxia and reoxygenation*

Significant increases in intracellular Ca^{2+} will trigger ROS production, which can alter mitochondrial function resulting in cell death.[26, 27] Recently it has been shown that stress activated proteins such as JNK, p38 as well ERK1/2 are activated under these conditions. [26, 27] In the current study, we measured changes in ERK1/2 expression in H9c2 cells subjected to anoxia-reoxygenation. Minimal effects were observed in cells subjected to 1h anoxia and 30min with less than 5% cell death in both control and 11,12-EET treated cells (Fig. 5.11A). However, ROS levels and expression of p-ERK1/2 was significantly higher in control

groups compared to 11,12-EET treated cells (Fig. 5.12A and 5.13A). Cells subjected to 6hr anoxia followed by 30min reoxygenation resulted in similar trends with less than 10% cell viability, increased ROS production and p-ERK1/2 expression (Fig.5.11B,5.12B and 5.13B). Increasing reoxygenation from 30min to 16hr following 6hr anoxia significantly increased cell death and slightly decreased ROS production in control groups which was attenuated by 11,12-EET (Fig. 5.11C and 5.12C). However, expression of p-ERK1/2 was higher in cells treated with 11,12-EET compared to controls (Fig. 5.13C). Evidence for a role of PI3K in EET-mediated protection was observed when co-treatment of PI3K inhibitors, wortmannin or PI-103 with 11,12-EET increased cell death compared to 11,12-EET alone (data not shown).

Consistent with changes in calcineurin activity observed in perfused hearts, a rapid and significant increase in intracellular Ca^{2+} load occurred following 1hr anoxia and 30min reoxygenation (Fig. 5.14A). This was attenuated by 11,12-EET but increased by co-treatment of cells with PI-103 or GLIB (Fig. 5.14A). Subjecting cells to 6hr anoxia and 16hr reoxygenation resulted in the dispersion of TMRE fluorescence from mitochondria in control groups (Fig. 5.16A), indicating dissipation of $\Delta\Psi m$ and suggesting loss of mitochondrial function. Co-treatment with 11,12-EET significantly slowed the anoxia-reoxygenation mediated loss of $\Delta\Psi m$, which was abolished when cells were co-treated with either GLIB (10µM) or PI-103(0.1µM) (Fig. 5.16A). Alterations to mitochondrial $\Delta\Psi m$ were not observed at the early injury time (1hr anoxia and 30min reoxygenation) suggesting that Ca^{2+} changes occurred upstream of mitochondrial dysfunction.

Consistent with increased cell death, 6hr anoxia and 16hr reoxygenation resulted in increased casapase-3 activity in control groups, which was attenuated with 11,12-EET (Fig. 5.15A). Co-treated with PI-103 or GLIB with 11,12-EET abolished the protective effect. Interestingly, experiments performed in calcium free media attenuated the activation of caspase-3 in all groups. Moreover, no difference in the casapase-3 activity was observed when cells were treated with 11,12-EET in either Ca^{2+} or Ca^{2+} free media (Fig. 5.15A). Thus, suggesting EET-mediated cardioprotection limits intracellular Ca^{2+} overload to mitochondrial function through activation of a PI3Kα-*pm*K_{ATP} dependent mechanism.

5.4 DISCUSSION

Recent evidence demonstrates that EETs can reduce myocardial damage against IR injury potentially through PI3K and *pm*K_{ATP} channels thereby limiting mitochondrial dysfunction.[5, 23, 28, 29] However, the intracellular mechanism through which EETs render this protection has not been elucidated. Mitochondria are the primary source of energy that fuels the contractile apparatus and act as key regulators of cell survival and death. Damage to these organelles, caused by IR injury, impacts the energetic state of the cell resulting in cellular functional dysfunction and death. In the current study, we demonstrate for the first time a central role for PI3Kα in EET-mediated cardioprotection. Moreover our data suggests that PI3Kα initiates a protective response involving *pm*K_{ATP} activation, which reduces Ca^{2+} overload and limits mitochondrial damage.

PI3Ks are members of a family of lipid kinases that phosphorylate the 3'-hydroxyl group of PIP and PIP_2 at the third position, to form PIP_2 and PIP_3.[16, 17, 30-32] PIP_2 and PIP_3 are important lipid messengers which activate many downstream kinases such as Akt, PKC and GSK-3β. PI3K activation has been demonstrated to regulate pro-apoptotic proteins thereby regulating mitochondrial function, reducing cell death and infarction size in IR injury.[17, 30-32] Previously, we and others, have shown activation of PI3K signalling plays an important role in EET-mediated cardioprotection, [6-8, 23] confirmed in the present study, using pan-specific inhibitors, such as wortmannin. While PI3Ks are classified into different classes, class I isoforms (class IA consists of α, β and δ isoforms, class IB consists of γ isoform) have a distinct role in regulating cardiac function.[17, 21, 30,

[32-34] Activation of PI3Kα is involved in regulating heart size and ventricular contractility.[19, 34, 35] While activation of PI3Kγ can have positive and negative effects on cardiac function depending on the pathological stimuli. For example, it was demonstrated that PI3Kγ activity is up-regulated in failing hearts [31] and that loss of PI3Kγ improves cardiac contractility.[21] In contrast, data suggests activation of PI3Kγ is beneficial for ischemic preconditioning.[20] The role of PI3Kβ in regulating cardiac function is still not known. In the current study we have illustrated that perfusion of PI3Kα inhibitor (PI-103)[36] did not affect the left ventricular function during the baseline, however following IR injury it abolished the EET-mediated improved left ventricular functional recovery. Whereas perfusing EET to PI3Kγ-/- hearts or treating hearts with a PI3Kβ inhibitor (TGX-221) failed to abolish the improved postischemic functional recovery. In addition, increased expression of Akt-p, a downstream kinase, was abolished with the PI3Kα inhibitor. Together these results suggest that EET-mediated cardioprotective signalling involves activating the PI3Kα isoform.

It is well established that opening of *pm*K_{ATP} channels improves postischemic ventricular functional recovery which can have a beneficial effect on mitochondrial function.[11, 12] We, and others, have previously demonstrated using non-specific pharmacological inhibitors that EET protective signalling involves activation of *pm*K_{ATP} channels.[4, 9, 10, 37] In the current study, EETs failed to improve the postischemic functional recovery in hearts from Kir6.2 -/-mice confirming the importance of *pm*K_{ATP} channels. Importantly, our data now

demonstrate that both the PI3K and *pm*K_{ATP} pathways are important for EETs and inhibition of either pathway eliminates the protective action.

PI3K metabolites, PIP_2 and PIP_3, can regulate ion channels, such as *pm*K_{ATP}, by binding to specific sites within the pore region of the K^+ channel.[10, 18, 38] Alteration to PIP levels during ischemia will impact function, therefore timing PI3K activation and specific subcellular localization is crucial to cardioprotection. Under basal conditions, the majority of PI3K remains inactive in the cytoplasm and levels of PIP_2/PIP_3 are relatively low.[64, 110] The speed and extent of PIP_2/PIP_3 accumulation depends upon the stressor and signal transduction pathway activated.[64, 110] Results from the current study suggest that EET-mediated activation of PI3Kα will initiate increased production of PIP_2/PIP_3, which will then interact with *pm*K_{ATP}.

The Kir6.2 subunit found in *pm*K_{ATP} channels is critical for channel-gating behaviour, regulating K^+ conduction and ATP-dependent inhibition.[10, 12, 39] The negatively charged phosphate head of PIP_2 and PIP_3 binds to these sites decreasing sensitivity to ATP.[18, 38] Interestingly, the C-terminal region of the Kir6.2 subunit contains overlapping binding sites for both PIP's and EETs.[10, 38, 40] Binding to these sites by either PIPs or EETs reduces channel sensitivity to ATP resulting in activation of *pm*K_{ATP} channels. Thus suggesting EETs might activate *pm*K_{ATP} channels by either direct binding to the C-terminal or through activation of PI3K. Bodiga et al demonstrated that EETs activate *pm*K_{ATP} channels independent of PI3K-mediated pathways, using the pan-specific inhibitor wortmannin, in cell culture systems. Thus suggesting that these two pathways

were separate in EET-mediated cardioprotection.[28] However, our data suggests that following IR injury, activation of PI3Kα and *pm*K_{ATP} channels are both required for EET-mediated cardioprotection. If these were truly independent pathways, we would have expected to observe increased postischemic functional recovery when EETs were perfused in hearts from Kir6.2-/- mice or following inhibition of PI3Kα. While EETs increased Akt-p in Kir6.2-/- mice following IR injury, suggesting activation of the PI3K pathway, we did not observe the improved postischemic recovery. Further support was observed when 11,12-EET mediated increased *pm*K_{ATP} channel currents were abolished by co-treatment with PI3K inhibitors, specifically PI3Kα inhibitor PI-103 (0.1 μM). As such, EET-mediated cardioprotection occurs through a PI3Kα dependent activation of *pm*K_{ATP} channels.

Alterations in metabolic status can lead to the activation of *pm*K_{ATP} channels which act to regulate the electrical-ionic gradient of the cell.[14, 15] Pharmacological and genetic studies have demonstrated *pm*K_{ATP} channels can protect the myocardium by preventing intracellular Ca^{2+} overload, thus limiting the activation of phosphatase enzymes, like calcineurin, and reducing contractile dysfunction.[14, 15, 24, 41] Recently, Marinovic et al demonstrated activation of *pm*K_{ATP} channels following oxidative stress prevents mitochondrial damage and apoptosis by decreasing intracellular Ca^{2+} overload.[42] In the present study, we demonstrate that increased cardiac EET levels will prevent IR induced calcineurin activity suggesting reduced Ca^{2+} overload. Moreover, the protective EET response prevented the mitochondrial localization of proapoptotic protein BAD

and subsequent activation of caspase-3 following IR injury. Consistent with these data, cell culture experiments demonstrate that EETs reduced anoxia-reoxygenation injury, limiting intracellular Ca^{2+} overload, caspase-3 activity and mitochondrial damage. This protective effect of EETs was abolished in Kir6.2-/- hearts and cells treated with either PI3Kα or *pm*K_{ATP} channel inhibitors, supporting the requirement of both pathways to decrease intracellular Ca^{2+} overload and maintain mitochondrial function. Taken together, these data proposes that EET-mediated cardioprotection limits intracellular Ca^{2+} overload and maintains mitochondrial function through activation of a PI3Kα- *pm*K_{ATP} dependent mechanism.

We postulate that the enhanced functional recovery observed with EETs entails modulation of both PI3Kα and *pm*K_{ATP} channels and that it mediates this through PI3Kα dependent activation of *pm*K_{ATP} channels. The data in the current manuscript is consistent with an important modulatory effect of CYP-derived eicosanoids in cardioprotection. The present study demonstrates that the protective mechanism of increased EETs limits intracellular Ca^{2+} overload and maintains mitochondrial function following IR injury. Taken together, these data suggest that manipulating EET levels may represent a novel therapeutic approach to management of ischemic heart disease in humans.

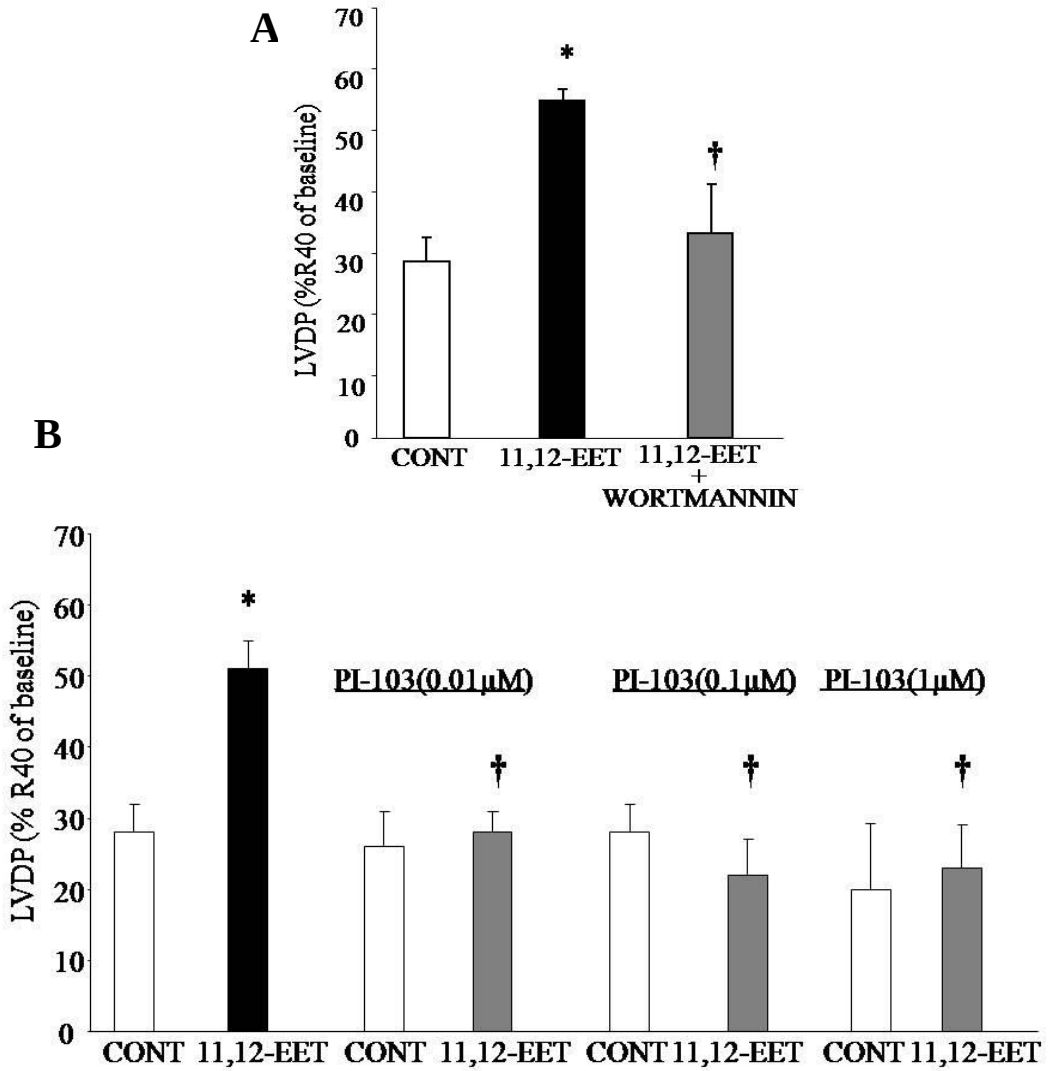

Fig. 5.1(A), Histogram of the functional recovery at 40min reperfusion expressed as percentage of baseline LVDP from C57BL6 hearts treated with vehicle, 11, 12-EET (1μM) or Wortmannin following 30min ischemia. Values represent mean±SEM, *n*=4-6 per group; *, *P*<0.05 vs. vehicle control; †, *P*<0.05 vs. 11, 12-EET treated group. (**B),** Histogram of the percentage of LVDP change at 40min reperfusion compared to baseline from the hearts perfused with vehicle, 11, 12-EET (1μM) or PI-103(0.01μM, 0.1μM or 1μM) following 30min of ischemia. Values represent mean±SEM, *n*= 5-6 per group; *, *P*<0.05 vs. vehicle control; †, *P*<0.05 vs. vehicle treated group.

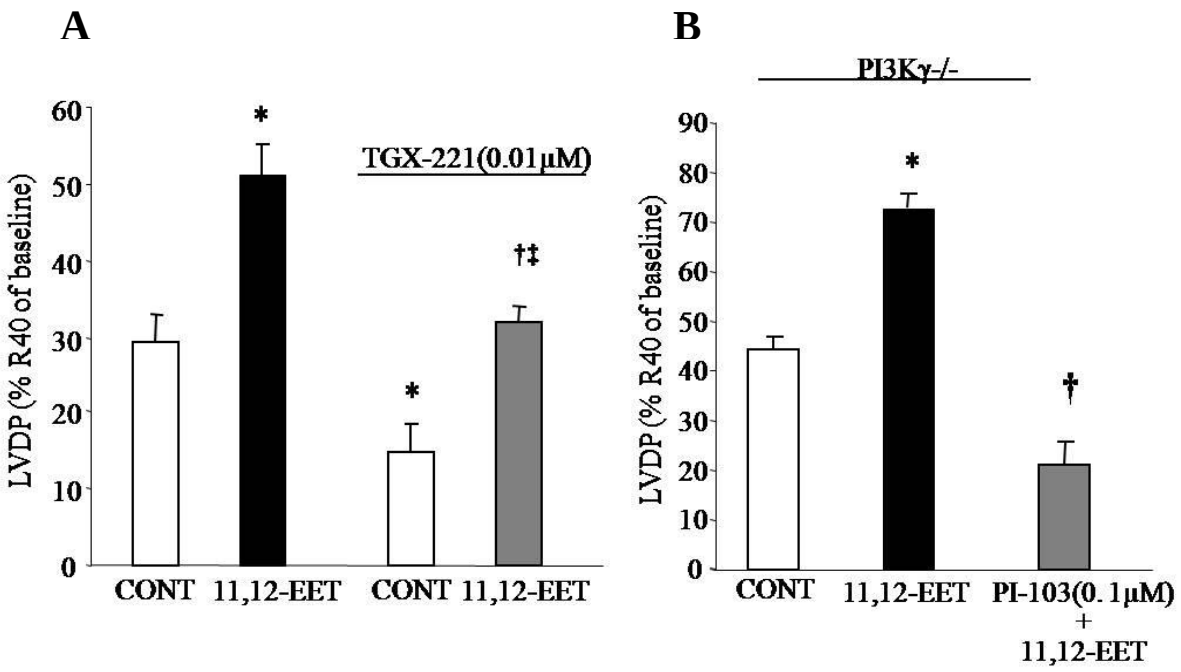

Fig.5.2 (A) , Histogram of the percentage of LVDP change at 40min reperfusion compared to baseline from the hearts perfused with vehicle, 11,12-EET(1μM) or TGX-221(0.01μM) following 30min of ischemia. Values represent mean±SEM, n= 3-6 per group; *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EET (1μM) treated group; ‡, P<0.05 vs. control group treated with TGX-221. **(B),** Histogram of the percentage of LVDP change at 40min reperfusion compared to baseline from the PI3Kγ-/- hearts perfused with vehicle, 11, 12-EET (1μM) or PI-103(1μM) following 30min of ischemia. Values represent mean±SEM, n= 3-6 per group; *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EET (1μM) treated group.

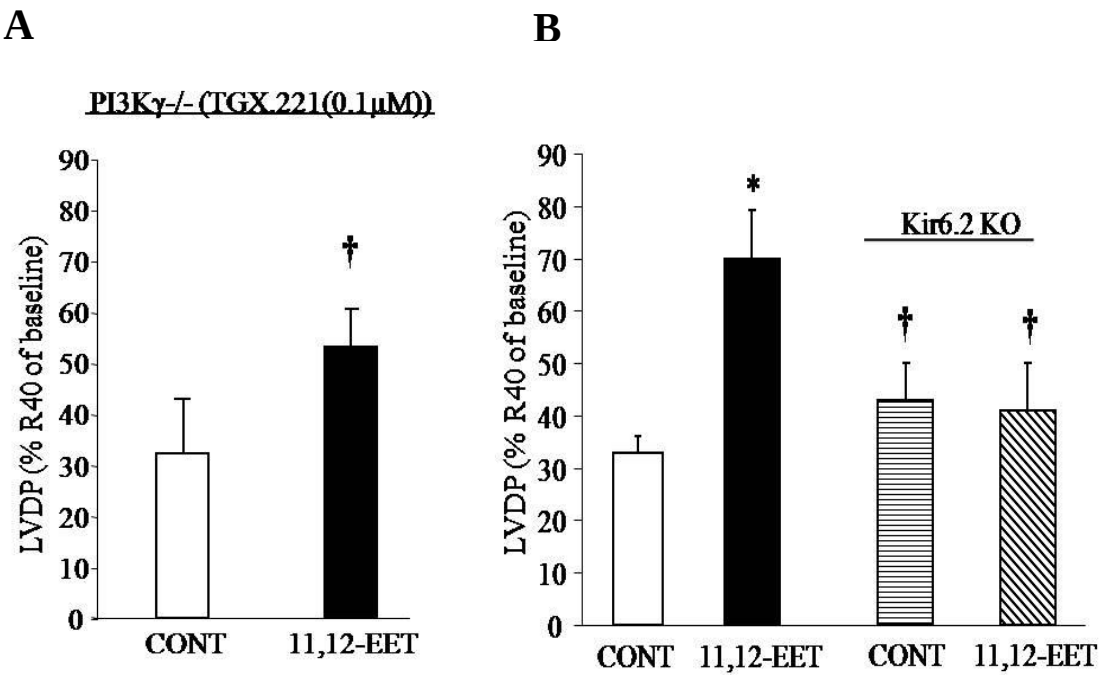

Fig. 5.3 (A), Histogram of the percentage of LVDP change at 40min reperfusion compared to baseline from the PI3Kγ-/- hearts perfused with 11, 12-EET (1μM) or TGX-221(0.01μM) following 30min of ischemia. Values represent means±SEM, n= 3-6 per group; †, P<0.05 vs. control group treated with TGX-221. **(B),** Histogram of the functional recovery at 40min reperfusion expressed as a percentage of baseline LVDP from WT and Kir6.2-/- hearts perfused with vehicle or 11,12-EET(1μM) following 20min ischemia. Values represent mean±SEM, n=6-11per group; *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EET treated group.

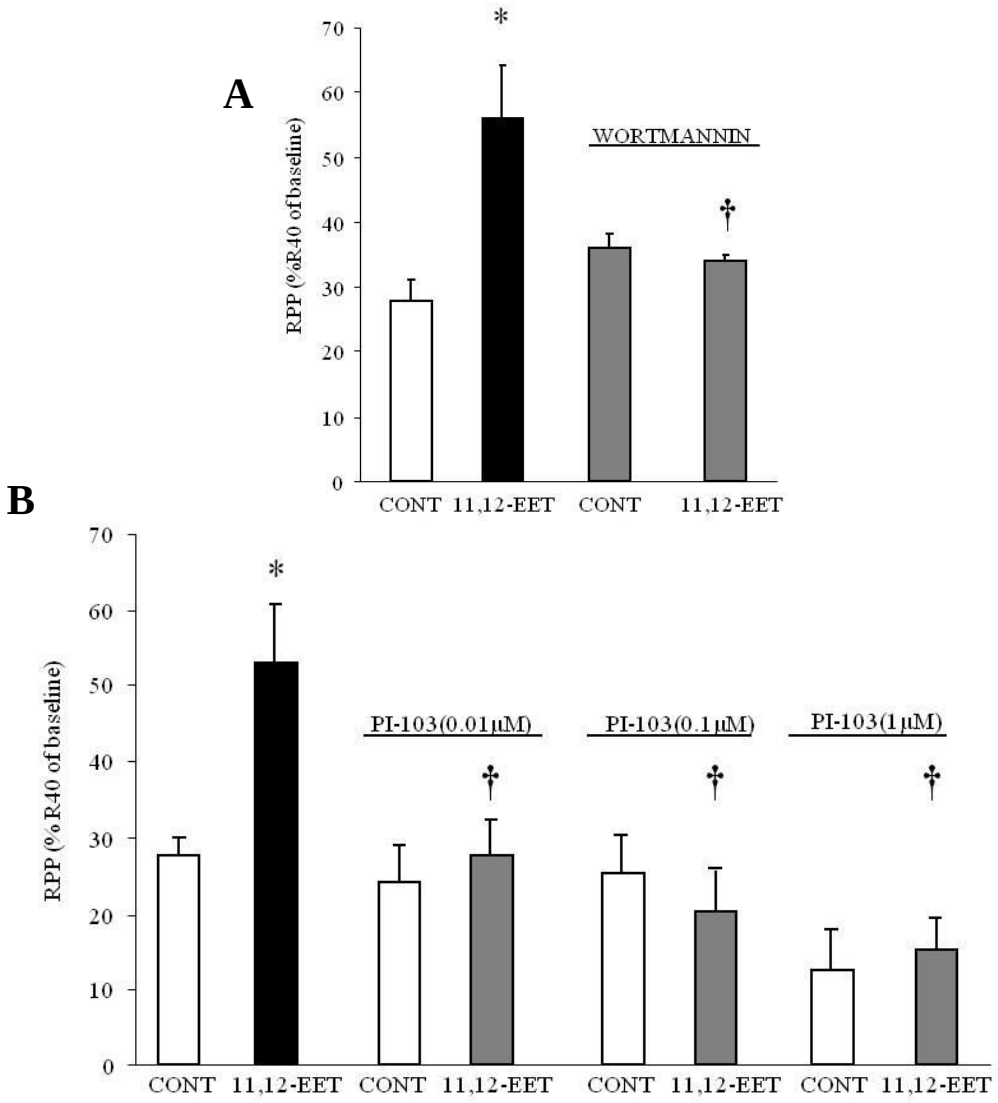

Fig. 5.4(A), Histogram of the functional recovery at 40min reperfusion expressed as percentage of baseline RPP from C57BL6 hearts treated with vehicle, 11, 12-EET (1µM) or Wortmannin following 30min ischemia. Values represent mean±SEM, *n*=4-6 per group; *, *P*<0.05 vs. vehicle control; †, *P*<0.05 vs. 11, 12-EET treated group. (**B),** Histogram of the percentage of RPP change at 40min reperfusion compared to baseline from the hearts perfused with vehicle, 11, 12-EET (1µM) or PI-103(0.01µM, 0.1µM or 1µM) following 30min of ischemia. Values represent mean±SEM, *n*= 5-6 per group; *, *P*<0.05 vs. vehicle control; †, *P*<0.05 vs. vehicle treated group.

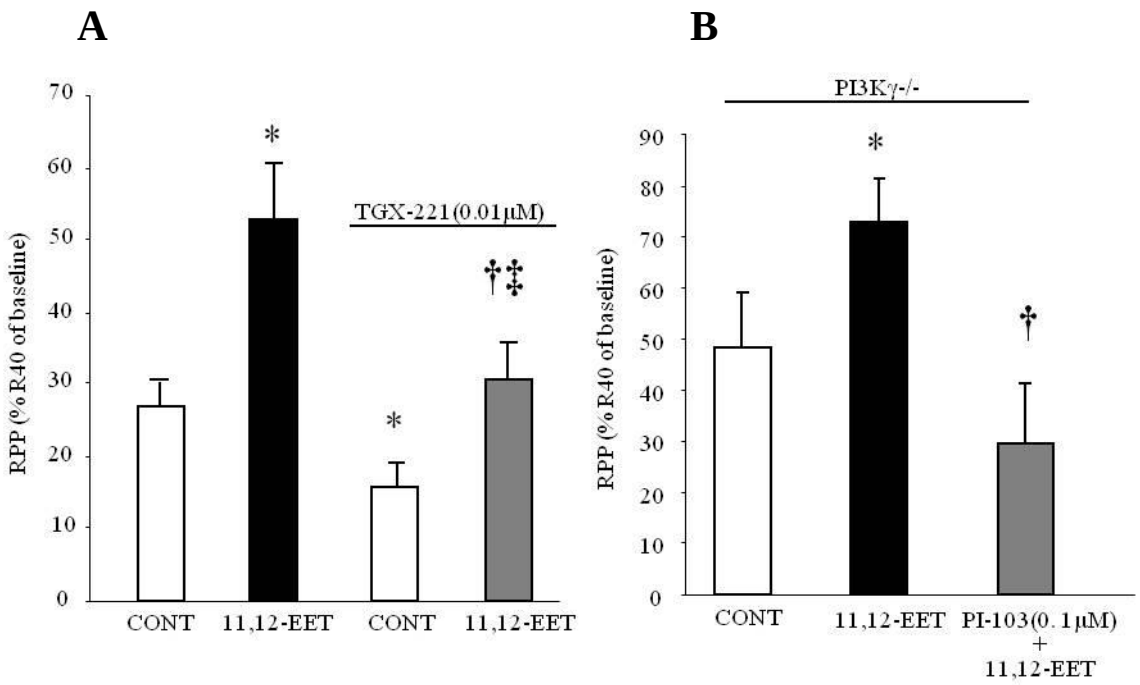

Fig.5.5 (A) , Histogram of the percentage of RPP change at 40min reperfusion compared to baseline from the hearts perfused with vehicle, 11,12-EET(1μM) or TGX-221(0.01μM) following 30min of ischemia. Values represent mean±SEM, n= 3-6 per group; *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EET (1μM) treated group; ‡, P<0.05 vs. control group treated with TGX-221. **(B),** Histogram of the percentage of RPP change at 40min reperfusion compared to baseline from the PI3Kγ-/- hearts perfused with vehicle, 11, 12-EET (1μM) or PI-103(1μM) following 30min of ischemia. Values represent mean±SEM, n= 3-6 per group; *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EET (1μM) treated group.

A

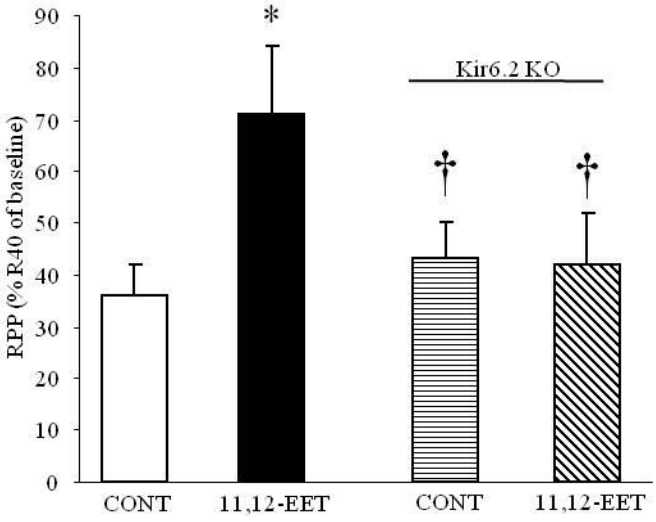

Fig.5.6 (A), Histogram of the functional recovery at 40min reperfusion expressed as a percentage of baseline LVDP from WT and Kir6.2-/- hearts perfused with vehicle or 11,12-EET(1µM) following 20min ischemia. Values represent mean±SEM, n=6-11per group; *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EETtreatedgroup.

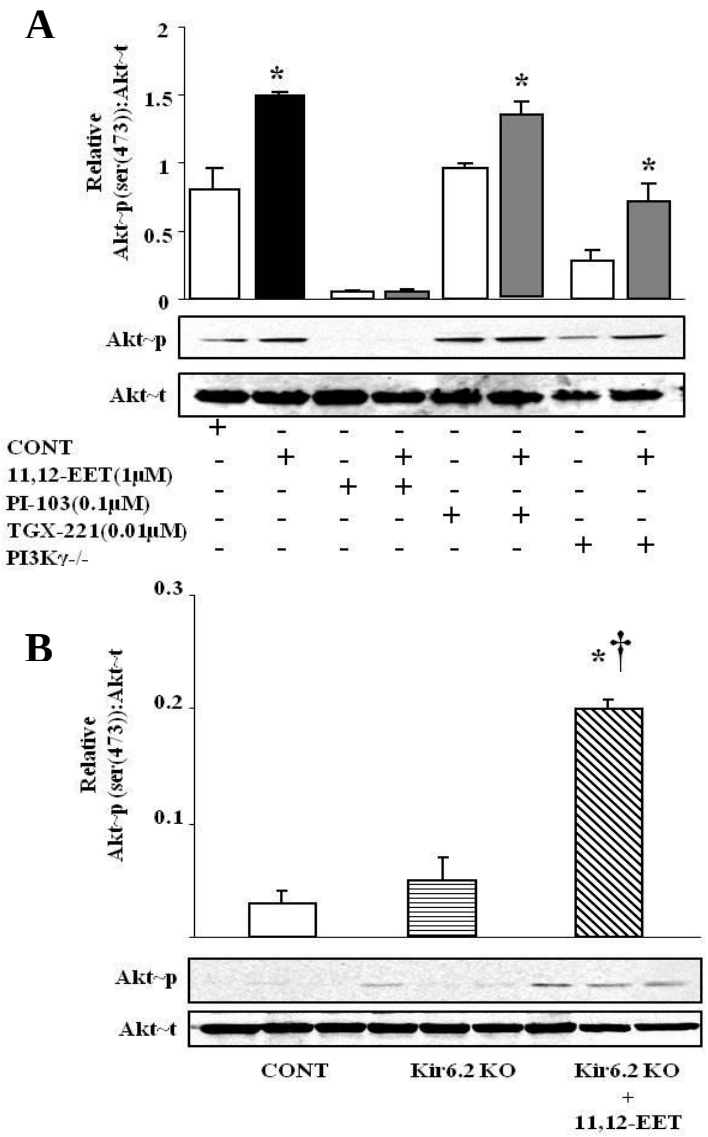

Fig. 5.7 (A), Immunoblot and densitometry showing the ratio of Akt-p to Akt-t expression from the C57BL/6 hearts treated with vehicle, 11,12-EET(1μM), PI-103(0.1μM) or TGX-221(0.01μM) and PI3Kγ-/- hearts treated with vehicle or 11,12-EET(1μM)**.** Values represent mean±SEM, *n*=3 per group; *, *P*<0.05 vs. control. (**B),** Immunoblot and densitometry showing the ratio of p-Akt to t-Akt expression from the C57BL/6 and Kir6.2 KO hearts treated with vehicle or 11, 12-EET (1μM)**.** Values represent mean±SEM, *n*=3 per group; *, *P*<0.05 vs. vehicle control; †, *P*<0.05 vs. vehicle treated Kir6.2 KO hearts.

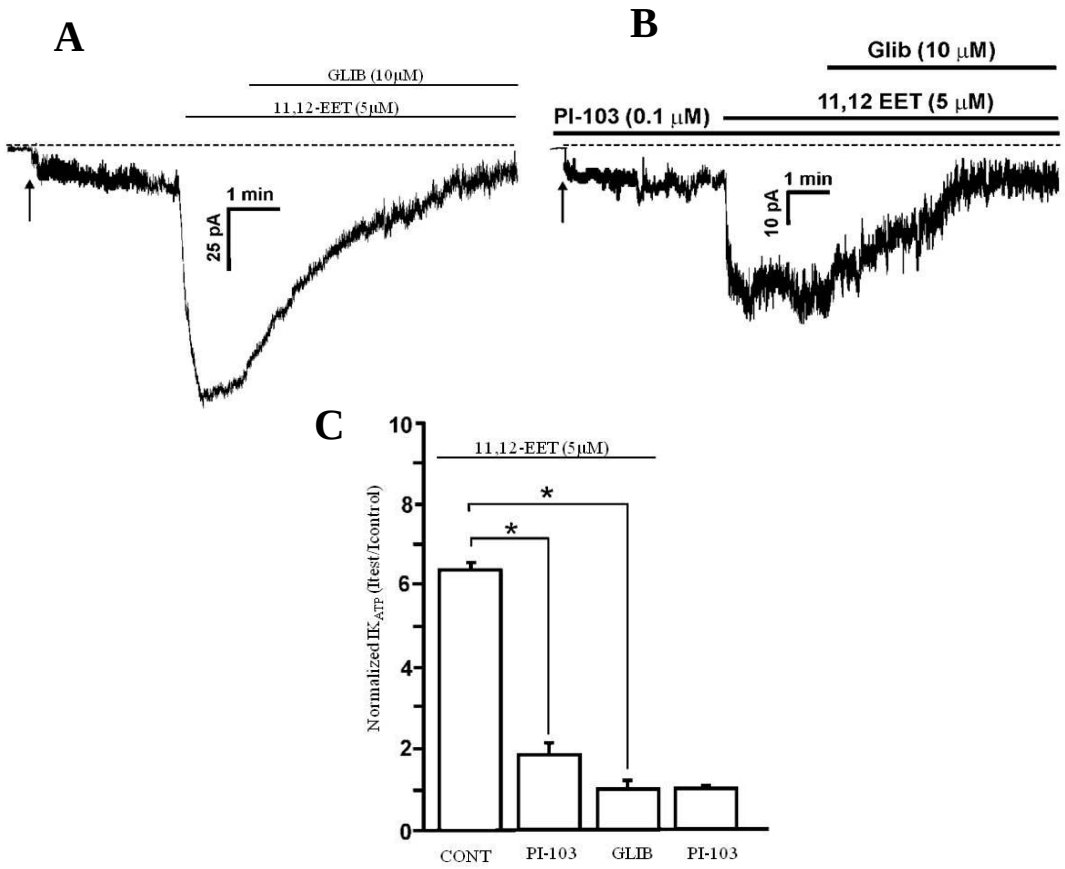

Fig. 5.8 (A), Representative whole-cell K_{ATP} channel current recordings illustrating activation by 11,12 EET and inhibition of the 11,12 EET effect by the GLIB (10 μM). The dashed line indicates the zero current level. The arrow denotes time of cell rupture and dialysis of the pippette solution used to elicit K_{ATP} channel currents. ***(B),*** Representative whole-cell K_{ATP} channel current recordings illustrating activation by 11,12 EET and inhibition of the 11,12 EET effect by the PI3Kμ inhibitor PI-103 (0.1 μM) and GLIB (10 μM). The dashed line indicates the zero current level. The arrow denotes time of cell rupture and dialysis of the pippette solution used to elicit K_{ATP} channel currents. **(C),** Grouped data from 5-7 recording in each group. *, $P < 0.05$ vs 11,12-EET treated group.

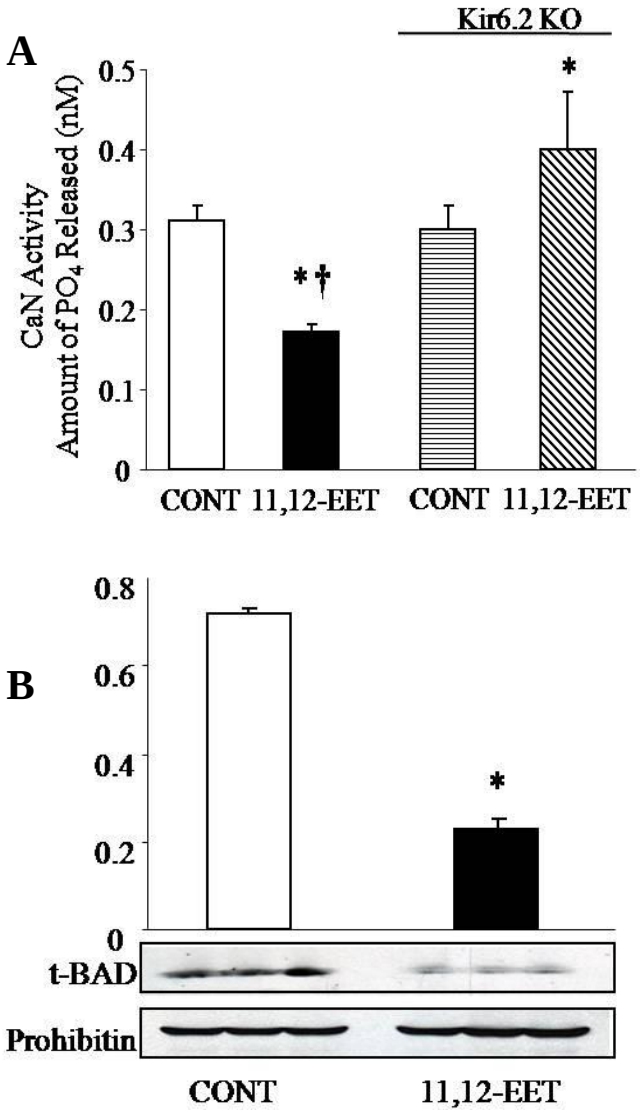

Fig. 5.9 (A), Quantification of calcineurin activity from the cytosol fraction of C57BL6 and Kir6.2 KO hearts perfused with vehicle or 11, 12-EET (1µM). Values represent mean±SEM, n=3per group; *, P<0.05 vs. vehicle control; †**,** P<0.05 vs. 11, 12-EET treated group. **(B)**, Immunoblot and densitometry showing the ratio of BAD-t to prohibitin expression from the C57BL/6 hearts treated with vehicle or 11, 12-EET (1µM)**.** Values represent mean±SEM, n=3 per group; *, P<0.05 vs. vehicle control.

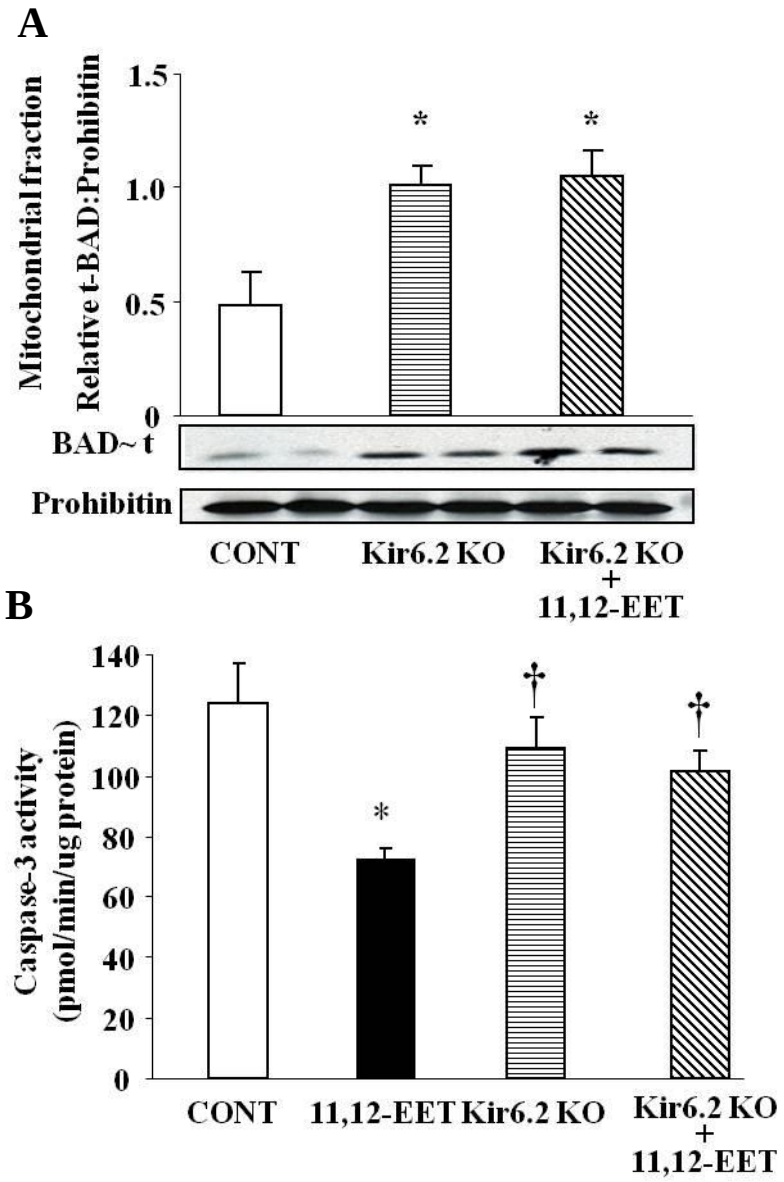

Fig. 5.10 (A), Immunoblot and densitometry showing the ratio of BAD~t to prohibitin expression from the C57BL/6 and Kir6.2 KO hearts treated with vehicle or 11,12-EET(1µM)**.** Values represent mean±SEM, *n*=3 per group; *, *P*<0.05 vs. vehicle control. **(B),** Caspase-3 activity in the cytosolic fraction of the C57BL6 or Kir6.2 KO hearts treated with vehicle or 11, 12-EET (1µM) and were subjected to IR injury**.** Values represent mean±SEM; n=3; *, *p*<0.05 vs. vehicle control.

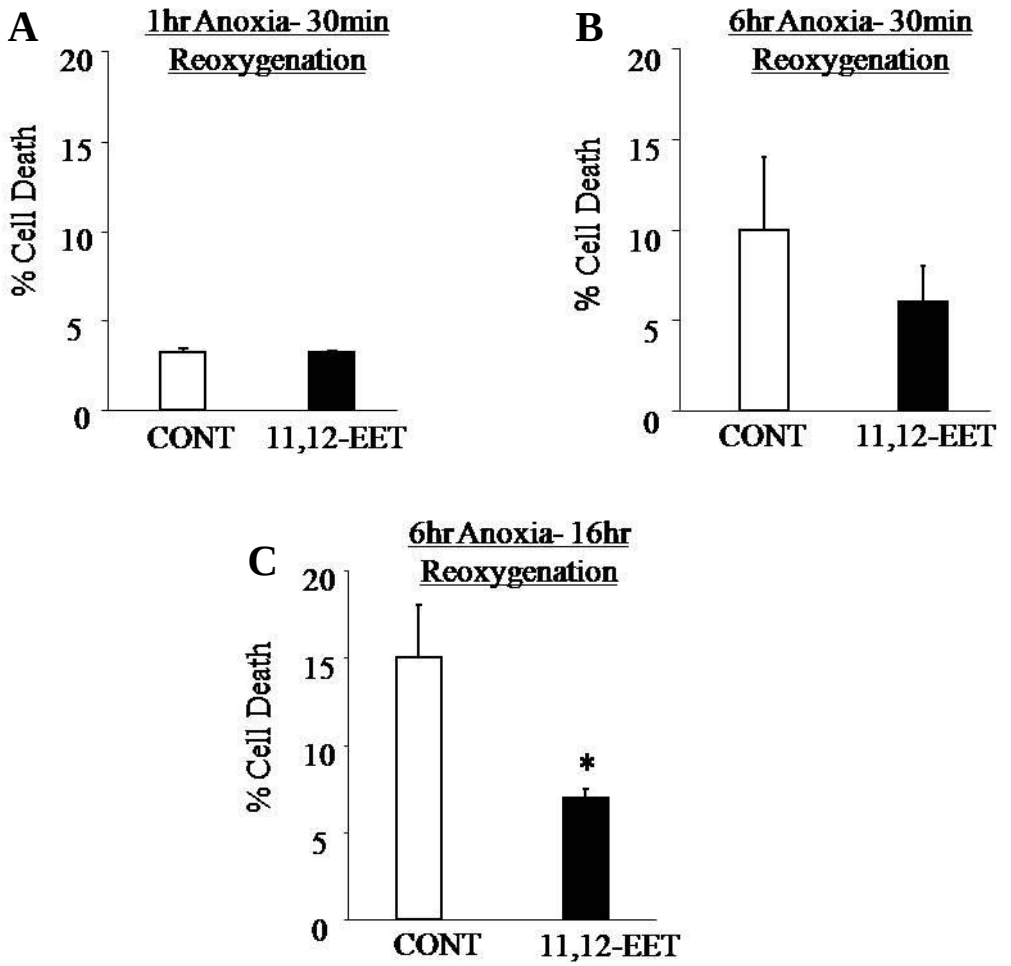

Fig.5.11 (A), Histogram representing the percentage of cell death between the vehicle and 11, 12-EET (1µM) treated groups following 1hr of anoxia and 30min reoxygenation**.** Values represent mean±SEM, n=4-6 per group *, P<0.05 vs. vehicle control. (**B),** Histogram representing the percentage of cell death between the vehicle or 11, 12-EET (1µM) treated groups following 6hr of anoxia and 30min reoxygenation**.** Values represent mean±SEM, n=3-4 per group *, P<0.05 vs. vehicle control. (**C),** Histogram representing the percentage of cell death between the vehicle or 11, 12-EET (1µM) treated groups following 6hr of anoxia and 16hr reoxygenation**.** Values represent mean±SEM, n=3-4 per group *, P<0.05 vs. vehicle control.

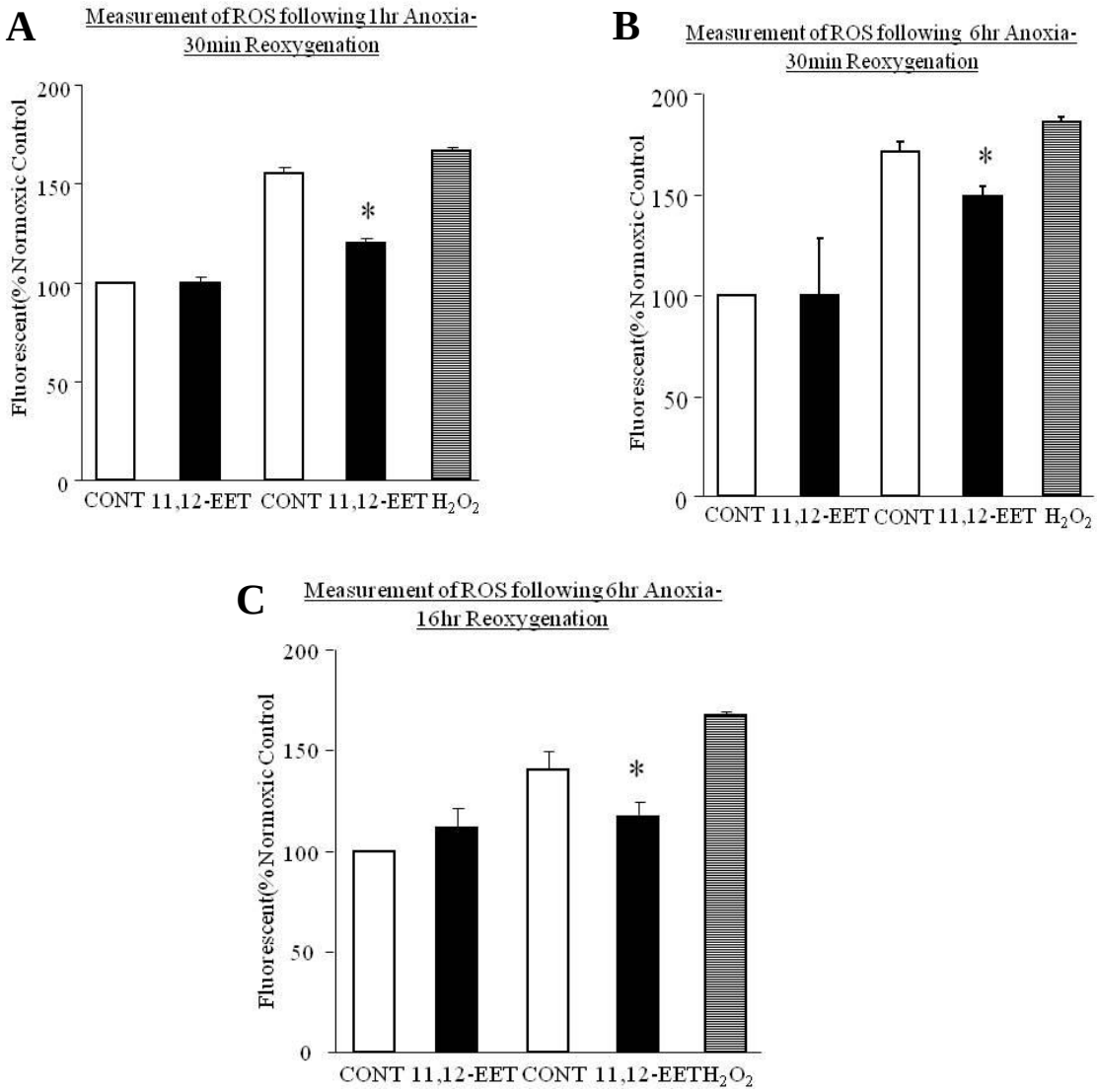

Fig. 5.12 (A), Levels of ROS generated by the vehicle or 11, 12-EET (1µM) treated groups following 1hr anoxia and 30min reoxygenation. Values represent mean±SEM, n=6-8per group; *, P<0.05 vs. vehicle control. **(B),** Levels of ROS generated by the vehicle or 11, 12-EET (1µM) treated groups following 6hr anoxia and 30min reoxygenation. Values represent mean±SEM, n=5-6per group; *, P<0.05 vs. vehicle control. **(C),** Levels of ROS generated by the vehicle or 11, 12-EET (1µM) treated groups following 6hr anoxia and 16hr reoxygenation. Values represent mean±SEM, n=5-6per group; *, P<0.05 vs. vehicle control.

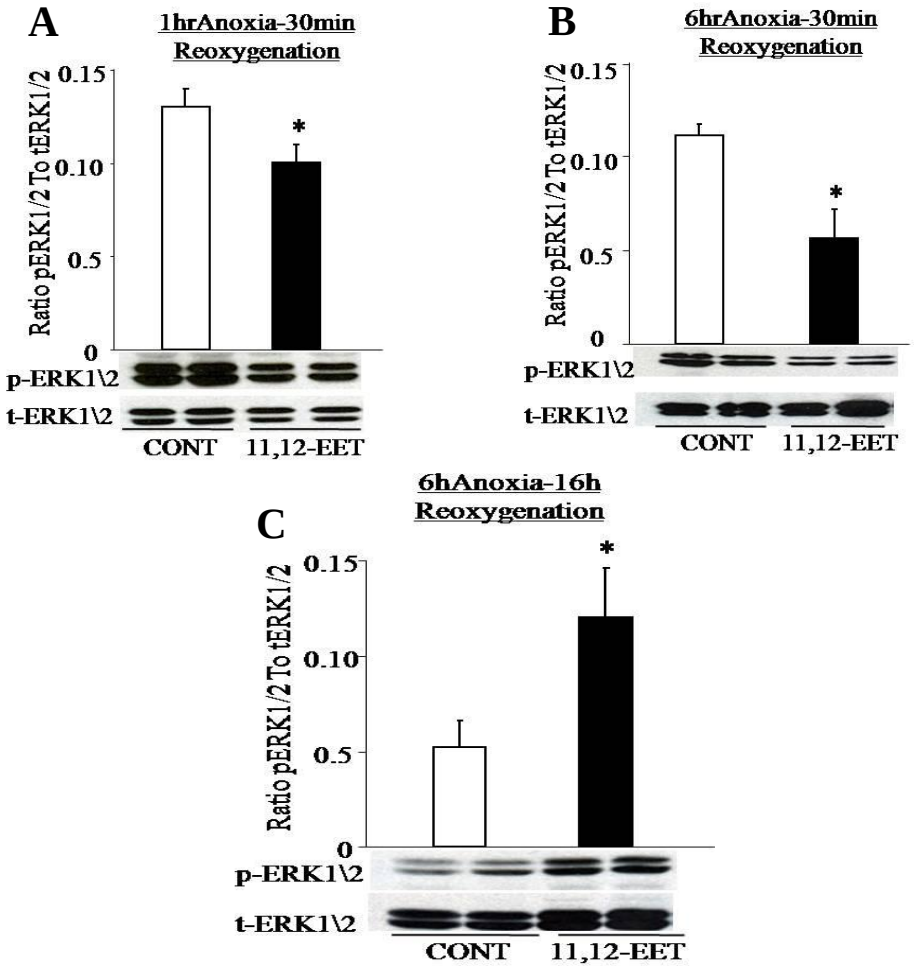

Fig. 5.13 (A), Immunoblot and densitometry showing the ratio of p-ERK1/2 to t-ERK1/2 expression from H9c2 cells treated with vehicle or 11, 12-EET (1µM) following 1hr anoxia and 30min reoxygenation**.** Values represent mean±SEM, n=4-6 per group; *, P<0.05 vs. vehicle control. **(B),** Immunoblot and densitometry showing the ratio of p-ERK1/2 to t-ERK1/2expression from H9c2 cells treated with vehicle or 11, 12-EET (1µM) following 6hr anoxia and 30min reoxygenation**.** Values represent mean±SEM, n=4-5 per group; *, P<0.05 vs. vehicle control. **(C)**, Immunoblot and densitometry showing the ratio of p-ERK1/2 to t-ERK1/2 expression from H9c2 cells treated with vehicle or 11, 12-EET (1µM) following 6hr anoxia and 16hr reoxygenation**.** Values represent mean±SEM, n=3; *, P<0.05 vs. vehicle control.

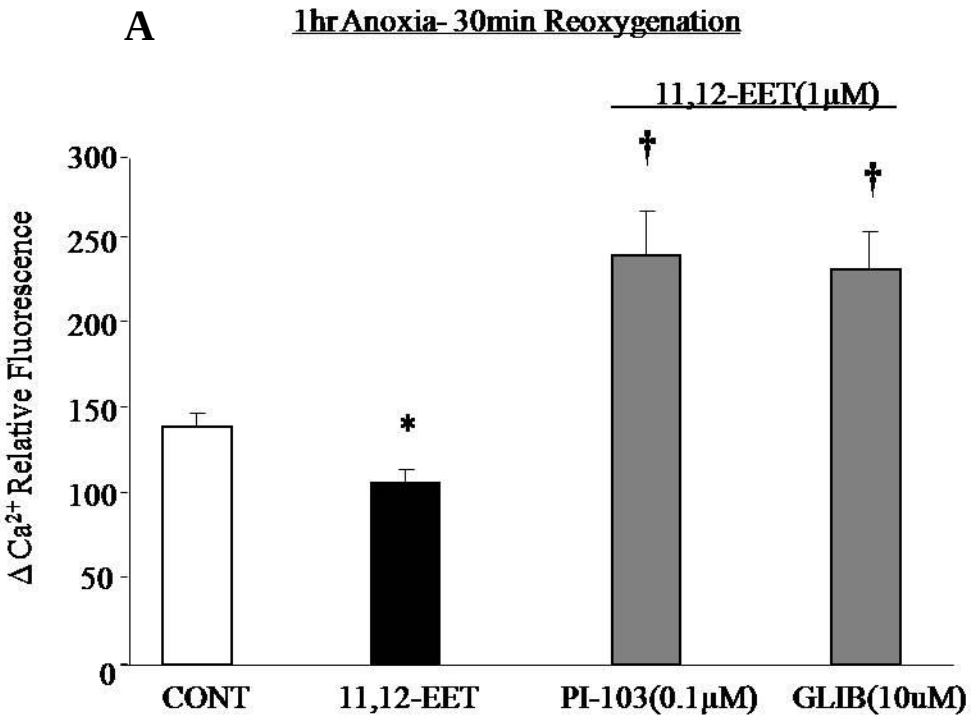

Fig. 5.14 (A), Histogram represents the amount of Ca^{2+} loaded inside the cells treated with vehicle, 11,12-EET(1μM), PI-103(0.1μM) or GLIB (10μM) following anoxia and reoxygenation. Values represent mean±SEM, n=30per group *, P<0.05 vs. vehicle control; †, P<0.05 vs. 11, 12-EETs (1μM) treated group.

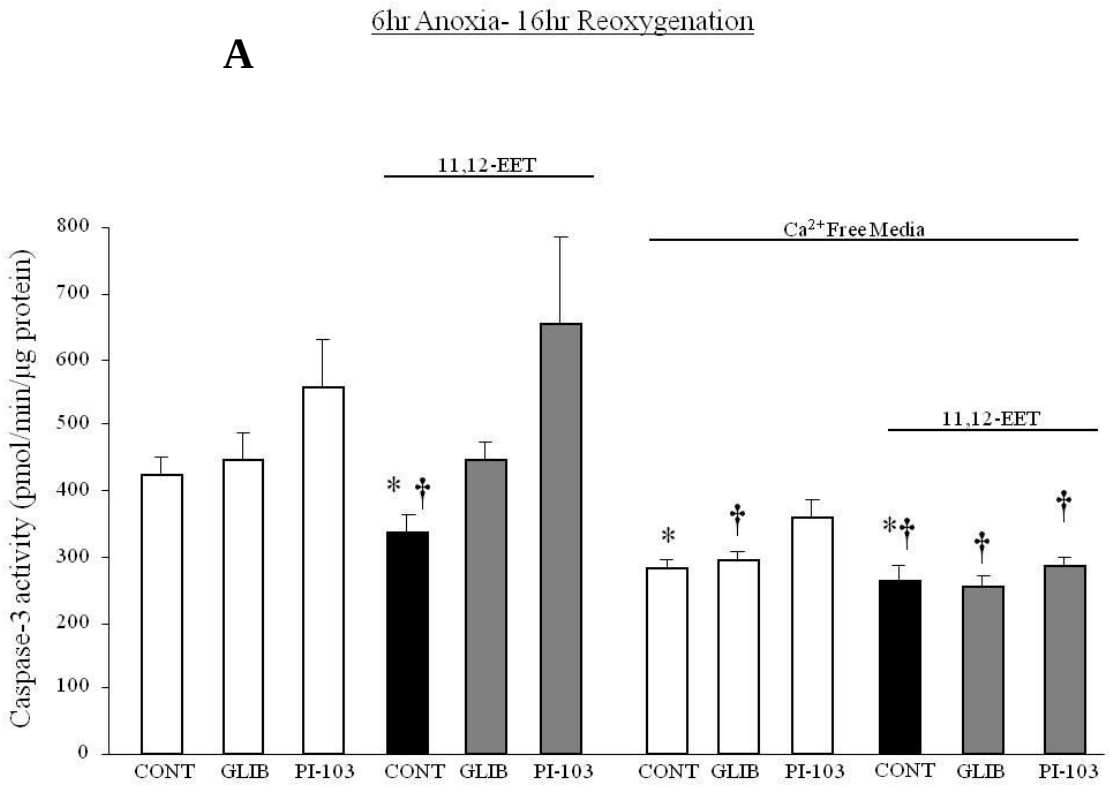

Fig. 5.15 (A), Caspase-3 activity in H9c2 cells using Ac-DEVD-AMC as substrate. Cells subjected to 6h anoxia and 16h reoxygenation were treated with vehicle, 11,12-EET(1µM) PI-103(0.1µM) or GLIB (10µM) in presence of regular or Ca^{2+} free DMEM media **.** Values represent mean±SEM; n=3; *, $p<0.05$ vs. vehicle control; †, $P<0.05$ vs. drug treated groups in presence of regular DMEM media.

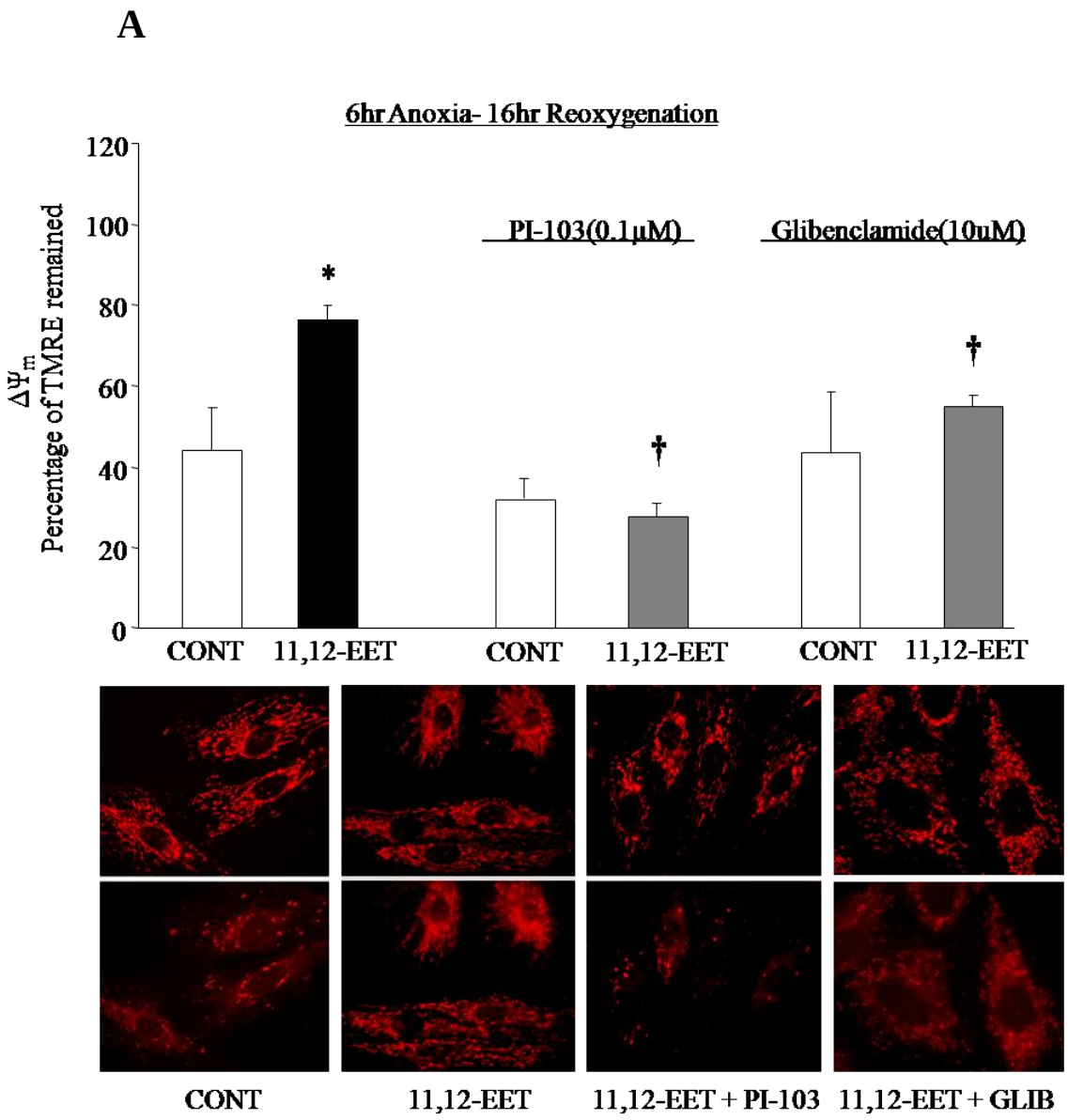

Fig.5.16 (A), Histograms and representative images show the percent of TMRE fluorescence lost in H9c2 cells treated with vehicle, 11, 12-EETs (1μM), PI-103(0.1μM) or GLIB (10μM)**.** Values represent mean±SEM; *n*=4-5, *, *P*<0.05 vs. vehicle control; †, *P*<0.05 vs. 11, 12-EETs (1μM) treated group.

	Preischemic				Postischemic (R40)			
	LVDP	dP/dt_{max}	dP/dt_{min}	HR	LVDP	dP/dt_{max}	dP/dt_{min}	HR
Vehicle Control	92±8	2677±338	-2223±213	333±20	21±2	606±39	-651±94	317±11
EET (1μM)	94±11	2443±243	-2143±207	313±25	51±10*†	1846±359*†	-1643±277*†	303±17
PI-103 (0.01M)	110±8	2926±159	-2618±186	291±4	29±8	965±262	-878±247	280±43
PI-103 (0.1μM)	93±16	2669±627	-2129±389	324±55	23±3	699±124	-579±120	355±58
PI-103 (1μM)	85±6	2185±238	-2154±278	300±66	13±7	395±208	-405±154	266±21
PI-103 (0.01μM)+ EET (1μM)	93±6	2945±307	-2588±252	353±28	27±3	873±90	-742±54	322±23
PI-103 (0.1μM)+ EET (1μM)	105±11	3000±367	-2509±249	330±33	23±7	644±148	-548±110	311±55
PI-103 (1μM)+ EET (1μM)	83±7	2428±338	-2207±269	321±14	18±4	521±120	-467±93	245±23
TGX-221(0.01μ M)	101±16	2827±349	-2441±347	338±25	15±6	590±50	-509±48	314±22
TGX-221(0.01μM)+ EET (1μM)	100±4	2610±20	-2322±35	348±29	29±6	974±52	-853±12	315±78
PI3Kγ KO	136±34	3044±70	-2747±578	265±53	58±17*	2371±167*	-2017±132*	267±28
PI3Kγ KO + EET (1μM)	112±13	3312±609	-2840±423	296±14	84±12*$^{\Phi\ddagger\dagger}$	2853±453*$^{\Phi\ddagger\dagger}$	-2272±309*$^{\Phi\ddagger\dagger}$	263±21
PI3Kγ KO + EET (1μM) + PI-103(0.1μM)	121±5	3020±104	-2456±64	287±9	25±6	711±172	-593±134	304±18
PI3Kγ KO + TGX-221(0.01μM)	125±18	3426±551	-2933±377	274±55	40±10	1405±612	-1262±580	305±34
PI3Kγ KO + TGX-221(0.01μM) + EET (1μM)	98±10	2437±302	-2125±232	329±16	57±4	1202±212	-1164±144	249±22

Table 5.1: Hemodynamic parameters measured in isolated-perfused hearts. Values represent mean±SEM, * $p<0.05$ vs Vehicle control, ‡ $p<0.05$ vs 11,12-EET treated hearts, † $p<0.05$ vs drug treated hearts, $^{\Phi}$ $p<0.05$ vs PI3Kγ KO hearts.

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CHAPTER-VI

General Discussion, Summary and Conclusion

General Discussion and Conclusion

6.1 Discussion

Data generated from this thesis provide insight into the mechanism of EET-mediated cardioprotection and identify nove pharmacological interventions that exploit this cardioprotection pathway. Importantly, this thesis demonstrates that requirement *pm*K_{ATP} channels and suggests that EET-mediated events involve activation of PI3Kα. Finally data presented hightlights the dependence of both both *pm*K_{ATP} channels and PI3Kα; moreover, that activation of PI3Kα is upstream of *pm*K_{ATP} channel activation.

Evidence has begun to identify the significance of cardiac cytochrome P450 monooxygenases toward heart function and protection; however, effects are dependent on the metabolites produced.[1-8] CYP epoxygenase metabolites, EETs, have protective effects within the cardiovascular system,[1, 2, 6, 9, 10] whereas other metabolites such as CYP hydroxylase products, 20-HETE have no protective or detrimental effects.[4, 5, 7, 10, 11] The cardioprotective response of EETs have been studied using the various approaches such as genetically altered mice, such as CYP2J2 Tr mice, sEH KO mice which lack sEH enzyme, and pharmacologically using EET antagonist, 14,15-EEZE, or the epoxygenase inhibitor, MS-PPOH. [1, 6, 12-14] Additional evidence comes from studies conducted with synthetic EETs with and without specific inhibitors.[4, 10, 15, 16] The data in the current thesis demonstrate alternative pharmacological methods to enhance EET levels (novel inhibitors of sEH) and function (EET mimetics, UA-8). Moreover data from the current study provide evidence for novel therapeutic agents and support the beneficial effects of

EETs. Further novel data from the thesis demonstrates that EETs reduce myocardial damage and render protection against IR injury through a mechanism involving activation of a PI3K pathway and *pm*K_{ATP} channel. Mitochondria are the primary source of energy that fuels the contractile apparatus of the myocytes and act as key regulators of cell survival and death. Damage to these organelles following IR injury has been shown to impact the energetic state of the cell resulting in cellular dysfunction and death.[21, 22] The data presented demonstrate that PI3Kα plays central role in EET-mediated cardioprotection. Moreover, it also suggests that PI3Kα initiates a protective response involving *pm*K_{ATP} activation which in turn reduces Ca^{2+} overload and limits mitochondrial damage.

EETs are chemically and metabolically labile compounds, that are rapidly metabolized, have a low solubility and storage issues limiting their use as therapeutic agents and pharmacological tools. Exogenously applied EETs or genetic alteration approaches which increase the intracellular EETs levels have a rapid cellular uptake and/or are metabolized by sEH resulting in reducing the half-life period from few minutes to seconds.[6, 17-19, 9, 20, 21] In order to elevate the levels of EETs, genetic modification techniques have been used in mice. Though these models have been successful they have had but had some limitations, such as clinical use and development of compensatory systems. Considerable interest has arisen in developing alternative methods to enhance the bioavailability of EETs.

sEH is a bi-functional enzyme with C-terminus hydrolase activity and N-terminus phosphatase activity.[22, 23] The effect of phosphatase on EETs is not known, as the hydrolase activity is responsible for conversion of EETs to DHETs.

Decreasing the hydrolysis of EETs by targeting the hydrolase component with pharmacological sEH inhibitors as an approach to elevate EETs has gained significant momentum in the research community.[22, 24, 25] Various sEH inhibitors have been developed that are based on urea, amide or carbamate groups.[22, 24, 25] The first generation sEH inhibitors synthesised contained epoxide groups that had only a transient inhibitory effect. [22, 23 31, 32] Ineffectiveness of these compounds encouraged development of other inhibitors with[23, 2623, 2623, 26] urea, carbamate and amide groups that demonstrated strong sEH inhibitory properties. However, these compounds are rapidly metabolized by amidases limiting their effectiveness.[23] To reduce their rapid metabolism, side chains were added, such as 1,3-disubstituted urea, carbamates and amides based sEH inhibitors.[27, 28] The second generation sEH inhibitors, 1,3-disubstituted urea, were reported to be very stable and potent sEH inhibitors.[28] Further development of various 1,3-disubstituted urea-based sEH inhibitors such as CDU (1-Cyclohexyl-3-dodecyl-urea), AUDA (12-(3-adamantan-1-yl-ureido) dodecanoic acid), AUDA-BE (butyl ester) and new generation inhibitors like *t*-AUCB (trans-4-[4-(3-adamantan-1-y1-ureido)-cyclohexyloxy]-benzoic acid) have also improved oral bioavailability and stability.[25, 29]

The present thesis characterized three novel pharmacological sEH inhibitors (BIX01960, BIX01961 and BIX01962) that are similar in potency (IC_{50}) to 1,3- disubstituted urea based established inhibitors like TUPS, AUDA and AUDA-BE. Perfusion with BIX01962 (IC_{50}= 15nM) improved postischemic left ventricular functional recovery and decreased infarct size. In contrast, while

BIX01960 (IC_{50}= 6.6nM) showed a similar protective effect as BIX01962 at lower concentrations, at a higher concentration (10 μM) it failed to improve functional recovery. Interestingly, in spite of being a very potent sEH inhibitor, BIX01961 (IC_{50}=6.4nM) failed to improve postischemic functional recovery. Data from these studies provide evidence that targeting sEH is a viable therapeutic approach of interacting with EET metabolism to improve postischemic functional recovery and reduce infarct size. This is in agreement with recently published studies using other sEH inhibitors against IR injury. [6, 25, 30] In spite of being very effective and potent therapeutic agents, many drawbacks have been reported with sEH inhibitors, such as rapid metabolism, low oral bioavailability or low water solubility.[24] It has been demonstrated that sEH inhibitors might shift EET degradation to other routes such as ß-oxidation, autooxidation and esterification, as well as increase EET oxidation.[31]

Structure activity relationship studies have demonstrated that EETs require a negatively charged group at the C-1 or need to be partially saturated with a double bond between 8, 9Δ-carbon to protect against autooxidation, as well as an epoxy group to possess EET functional effects.[31] The epoxy group can be replaced with epoxy bioisosteres like urea, thiourea and oxiamide, which have sEH inhibitory properties but still maintain functional activity protecting against sEH hydrolysis. [31, 32-34] Recently, Falck *et al*. developed UA-8 a structural analog of EET that is partially saturated, making it less prone to oxidation, and contains a 1,3-disubstituted urea replacing the epoxy group.[31] UA-8 mimics the vasodilator properties of EETs at much lower concentrations (100-fold) compared to

synthetic EETs and exhibits resistance to oxidation in preconstricted bovine arteries.[31] UA-8 inhibits sEH at nanomolar concentrations (IC_{50} 46nM) similar to that of urea based sEH inhibitors.[31] UA-8 is susceptible to ß-oxidation and esterification which could possibly reduce its bioavailability. However, increased resistance to autooxidation and degradation by sEH enzyme provides UA-8 a significant advantage compared to other sEH inhibitors leading to a longer duration of its protective action.[31] In the present study, we demonstrated that hearts perfused with UA-8 at concentrations as low as 100 nm showed significant improvement in LVDP, prevented the loss in cardiac contractile function, similar to the cardioprotection elicited by EETs (1µM) or sEH null mice. Moreover, UA-8 significantly reduced the amount of irreversible injury or infarction compared to control and EET-treated hearts. Thus, demonstrating UA-8 has better potency compared to EETs alone, is most likely due to its increased stability.

Although UA-8 improved cardiac function after IR injury in mice, an important question remained regarding the relative contribution of the EET mimetic aspect versus the sEH inhibitor aspect of UA-8 to the cardiac protection. Experiments were performed with another EET analog UA-7, which is structurally similar to UA-8 but has very little sEH enzyme inhibition activity IC_{50} of 58712nM. Nevertheless UA-7 improved recovery following IR injury, data suggest suggest that the protective effect of UA-8 is attributed to its EET mimetic properties as opposed to sEH inhibition[313131]. This finding was further supported when UA-8 and UA-7 improved postischemic recovery and this effect was attenuated by the EET-antagonist, 14, 15-EEZE and no change in their protective

effect when co-perfused with MSPPOH. Thus, the data presented in chapter 4 confirms that targeting EET-dependent effects to limit IR injury with pharmacological interventions to alter the half life and intracellular concentrations of EETs are a good approach.

While it has been known that elevation of EET levels render cardioprotective and protect the heart against IR injury but the exact mechanism through which it the affects the cardiovascular physiology and function remains unknown.

Ischemic events are known to affect the metabolic profile of the cell, alter ionic homeostasis, ventricular contractility and affect EKG parameters, finally leading to cardiac arrest and death.[35-37,38] Activation of ATP sensitive potassium channels are known to render protection aganist stress-induced injury. [28, 39, 40 41, 42, 10, 40, 43] There are two types of potassium channels, mitoK_{ATP} and *pm*K_{ATP} channels, based on where they are expressed. Activation of *pm*K_{ATP} channels are shown to hyperpolarize the cell, shorten the APD and improve contractility following the injury.[44-47] While the exact structural identity of the mitoK_{ATP} remains unknown, evidence suggests that activation of the channel provides protection. Studies demonstrate that following IR injury EETs are known to render protection through activation of these channels. [28, 39, 40 41, 42, 10, 40, 43] However, it is still not clear whether mitoK_{ATP} and *pm*K_{ATP} channels are important for EETs mediated cardioprotection. Data from Chapter 2 and 3 demonstrated that perfusion with *pm*K_{ATP} specific (HMR1098) and/or non-specific inhibitors (glibenclamide) abolished the cardioprotective effects observed

in hearts from CYP2J2 Tr mice and WT hearts perfused with EETs or the sEH inhibitor, BIX01962 . Next, data present in Chapter 3, [1, 6, 481, 6, 481, 6, 48] using an *ex vivo* EKG model demonstrated that EETs attenuated ischemic injury-mediated EKG abnormalities through activation of *pm*K_{ATP} apart from improving ventricular contraction and relaxation. Baseline EKG parameters were similar in EET-treated or CYP2J2 Tr mice and that significant prolongation in QT interval and STE occurred to a greater extent in WT mice following an ischemic event. Moreover, the reduction in abnormal EKG waveforms was observed in CYP2J2 Tr or EET-perfused hearts. Further more these protective effects were inhibited when these hearts were perfused with *pm*K_{ATP} channel inhibitors suggesting that elevated levels of EETs attenuate changes in measures of electrocardiogram parameters (QT interval and STE) apart from improving ventricular function following IR injury through activation of these channels. Moreover, our data demonstrate that there was no difference in protein expression of *pm*K_{ATP} among the treated and control groups. Finally, data more directly addressing the role of *pm*K_{ATP} in EET-mediated cardioprotection was presented in Chapter 5, in which hearts from Kir6.2 KO mice were perfused with EETs and subjected to IR injury. EETs failed to improve postischemic left ventricular functional recovery in Kir6.2 KO mice. Together data in this thesis clearly suggest that *pm*K_{ATP} channels are important for EET-mediated cardioprotection and has a role in EET-mediated control over EKG abnormalities.

Interestingly when WT hearts treated with EETs or hearts from CP2J2 Tr were co-perfused with H89, a PKA inhibitor, the improved ventricular

repolarisation seen in these hearts was abolished. Supporting these data, increased plasma membrane expression of activated PKAα regulatory II subunit was observed in CYP2J2 Tr hearts. Expression of activated PKAα regulatory II subunit in the plasma membrane fraction suggests the involvement of PKA in ion channel activity. Lu *et al* have demonstrated using patch clamp technique that *pm*K_{ATP} channels in vascular region but not the cardiac *pm*K_{ATP} are activated by EETs via cAMP-PKA mediated event.[40] Recently, Ke *et al* [39] demonstrated that CYP2J2-derived EETs affect cardiac APD and $I_{to,peak}$ via a cAMP-dependent mechanism suggesting EETs increase intracellular cAMP production triggering a cAMP-PKA dependent phosphorylation of Kv4.2 channels resulting in enhanced maximal peak transient outward currents. The fact that inhibitors of this pathway completely abolish the EET mediated improved postischemic ventricular repolarization suggests that, PKA-dependent signal also might play a potential role in EET-mediated cardioprotection. However, the role of this pathway in improving ventricular function following IR injury is unknown

Data from our laboratory and others have demonstrated that EET-mediated cardioprotection involves activation of a PI3K dependent pathway indicating more than one potential protective mechanism.[6, 43, 49,43, 49-51] PI3Ks are members of a family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphoinositides[52, 53] producing phosphoinositol-3, phosphate (PIP), phosphoinositol-3, 4, diphosphate (PIP_2), phosphoinositol-3, 4, 5-triphosphate (PIP_3). PIP's are important lipid messenger molecules; these are shown to regulate the localization and function of multiple proteins such as Akt and ion channels.[52-

[55,56]Enhanced activation of PI3Ks and its mediated signalling pathways involving many downstream kinases in IR are known to reduce cell death and infarct size.[52, 53, 56] Consistent with previous studies in Chapter 5 data presented demonstrate that hearts perfused with EETs following IR injury have increased expression of activated Akt. When these hearts were treated with a pan-specific PI3K inhibitor wortmannin the improved recovery was abolished confirming PI3K survival signalling is important for EET-mediated cardioprotection.[49-51, 57]

PI3Ks exist in various classes (class-I, -II and -III) based on mode of structure and activation.[52, 53] Out of all classes, class-I PI3Ks are well studied and are expressed ubiquitously. [52, 53] Class I isoforms (class IA consists of α, β and δ isoforms, class IB consists of an γ isoform) have a distinct role in regulating cardiac function.[53, 58-62] Activation of PI3Kα isoform is shown to be involved in regulating heart size.[59, 60, 62] Activation of PI3Kγ is shown to have positive and negative effects on cardiac function depending on the pathological stimuli.[63] For example, it was demonstrated that PI3Kγ activity is up-regulated in failing hearts [64] and that loss of PI3Kγ improves cardiac contractility.[59] In addition to these findings suggest that activation of PI3Kγ is required for ischemic preconditioning.[58] The role of PI3Kβ in regulating cardiac function is still not known. While EETs are known to render cardioprotection through activation of PI3K, the specific class and isoform involved in the EET protective mechanism is unknown. Most PI3Ks studies are often conducted by using non-specific inhibitors, like wortmannin, making it difficult to differentiate which isoform is involved. Data presented in Chapter 5 demonstrated that the class-I specific

PI3Kα inhibitor (PI-103, 10nM blocks only the PI3Kα isoform)[65, 66] abolished EET-mediated prtotection suggesting a role for PI3K's from class-I in the response. This finding was supported when hearts from PI3Kγ-/- mice perfused with EETs or the PI3Kβ inhibitor (TGX-221) failed to abolish the improved postischemic functional recovery. Increased expression of p-Akt, a downstream kinase, was also abolished when hearts were perfused with PI3Kα inhibitor. Together these results suggest that EET-mediates cardioprotection through activation of PI3K, more specifically the PI3Kα isoform from class-I, in addition to *pm*K_{ATP} channels.

Data presented in the thesis clearly suggest that EET-mediated cardioprotection involves both *pm*K_{ATP} channels and a PI3Kα–dependent pathway; however, the specific intracellular signaling mechanism(s) determining if these are dependent or independent pathways remains unclear. Downstream targets of PI3K, such as PIP's are known to modulate the activity of different K^+ channels such as *pm*K_{ATP}. These products are known to bind to specific sites within the pore region of the K^+ channels and regulate its activity.[55, 56, 67, 68] The Kir6.2 subunit found in *pm*K_{ATP} channels is the pore forming unit of the channel and is important for regulating K^+ conductance and ATP-dependent inhibition.[45, 68, 69] The negatively charged phosphate head of PIP's are also shown to bind certain sites (170 to 201) on the C-terminal region of the subunit and decrease the sensitivity to ATP leading to increased channel activity.[56, 67] Interestingly, the C-terminal region of the Kir6.2 subunit also contains binding sites for EETs (185 to 201) which overlap with PIP's binding site.[67, 68, 70] Studies have demonstrated that

EETs bind to these sites, which are similar to that of PIP's and reduce channel sensitivity to ATP resulting in activation of *pm*K_{ATP} channels. These findings suggest EETs might activate *pm*K_{ATP} channels by either direct binding to the C-terminal or through activation of PI3K. If these two pathways, *pm*K_{ATP} and PI3K, were independent EET-mediated mechanisms we would expect to observe, at least partially improved postischemic functional recovery when EETs were administered to hearts from either Kir6.2KO mice or following inhibition of PI3Kα. Inhibition of either pathway completed blocked EET-mediated protection suggesting these two pathways are dependent. Interestingly, evidence in Chapter 5 demonstrated hearts from Kir6.2KO mice perfused with EETs had increased p-Akt but no improved functional recovery. Furthermore, patch clamp experiments revealed myocytes treated with the PI3Kα inhibitor PI-103, blocked EET activation of *pm*K_{ATP} channels. Therefore, the data demonstrates that both *pm*K_{ATP} channels and PI3Kα are required for EET-mediated protection and PI3Kα is upstream of *pm*K_{ATP} channel activation.

An important question addressed in the thesis is how and/or what downstream pathways do EETs affect that preserve cardiovascular physiology and function following IR injury. Following oxidative stress any alteration in metabolic status of the cell leads to increase in intracellular Ca^{2+} overload. This Ca^{2+} accumulation has been implicated in intiation of arrythmias, activation of phosphatase enzymes, like calcineurin, pro-apoptotic proteins, mitochondrial damage, reducing contractile dysfunction and cell death.[71-74] The activation of *pm*K_{ATP} channels has shown to regulate the electrical-ionic gradient of the cell

following IR injury.[73, 75, 76] Pharmacological and genetic studies have demonstrated that activation of these channels protect the myocardium by regulating intracellular Ca^{2+} overload, thus limiting the activation of phosphatase enzymes, like calcineurin, pro-apoptotic factors and reduces contractile dysfunction.[71-74] Recently, Marinovic *et al* demonstrated activation of *pm*K_{ATP} channels following oxidative stress prevents mitochondrial damage and apoptosis by decreasing intracellular Ca^{2+} overload.[77, 78] In Chapter 5, data demonstrated that following IR injury hearts treated with EET had decreased Ca^{2+} activated calcineurin enzyme activity. Moreover, mitochondrial localization of proapoptotic protein BAD and subsequent activation of caspase-3 following IR injury was reduced in these hearts. Interestingly these protective effects of EETs were abolished in Kir6.2KO hearts linking *pm*K_{ATP} channels. Previously, data from our laboratory showed that following oxidative stress EETs provided cytoprotection by slowing or preventing the opening of mPTP.[79] Following IR injury this rise in intracellular Ca^{2+} concentrations is known to stimulate the opening of the mPTP.[80-85] Together with our previous studies, data from this thesis suggest that following IR injury, EET-mediated protection limits intracellular Ca^{2+} overload and maintains mitochondrial damage.

Results from cell culture experiments presented in Chapter 5 provide supporting evidence by demonstrating that EETs reduce anoxia-reoxygenation mediated injury, limit intracellular Ca^{2+} overload and prevent mitochondrial damage which all resulting in a reduction in cell death. This protective effect of EETs was abolished when the cells were treated with PI3Kα and *pm*K_{ATP} channel

inhibitors. These data suggest that EET-mediate cardioprotection occurs by limiting intracellular Ca^{2+} overload and preserving mitochondrial function through activation of a PI3Kα-*pm*K_{ATP} dependent mechanism.

6.2 Summary and Conclusion

IR injury remains the main underlying cause of morbidity and mortality in patients with ischemic heart disease. Despite the large amount of research focused on developing potential therapies, there are very few effective therapies available. Current therapy focuses on tissue reoxygenation, while effective, contributes to reperfusion injury. Damage of key organelles like mitochondria is recognised to be an important contributor for the pathogenesis of cardiovascular IR injury. It has been demonstrated that EET-mediated cardioprotective responses render protection following IR injury, but there is still considerable controversy regarding the specific intracellular mechanisms involved.

The data from this thesis identifies a novel cellular protective mechanism for EET induced protection through activation of PI3Kα-*pm*K_{ATP} channel dependent mechanism. This study provides a better understanding of how certain EETs regulate mitochondrial function and mediate protection against IR injury. Further data obtained from experiments completed using a synthesized EET analog, UA-8, were consistent with the results using EETs and may be a novel therapeutic agent to prevent cardiac injury caused by IR injury.

Though in the current thesis we have focused on the cardiac role of EETs against IR injury, the cellular signalling mechanisms identified can be applied to other tissues and diseases. Together, the protective mechanism of EETs

demonstrated in this thesis may direct and help in amalgamating the ongoing research in the development of novel therapeutic options for ischemic heart disease.

Future directions

Evidence and results from the present thesis demonstrate that both PI3K and *pm*K_{ATP} channels play a significant role in EET-mediated protection. Thus it is postulated that a PI3K specific isoform, PI3Kα, initiates protective signalling upstream of *pm*K_{ATP}. However further experiments should be conducted to examine and better understand the link between PI3Kα-*pm*K_{ATP} in producing cadioprotection:

1. Experiments to investigate how EETs can activate PI3Kα.

Further validation of this pathway could be assessed using genetically altered mice PI3Kα (dominant-negative) mice. The timing and subcellular localization of downstream events such as measuring PIP_2/PIP_3 levels, Akt and GSK3-β could be determined, PI3K activity is crucially controlled by protein-protein interactions, p110 catalytic subunits bind directly to p85 regulatory subunits. Activation of numerous receptor and non receptor tyrosine kinases (RTK) results in binding of p85 subunits along with p110 to the receptor in turn leading to activation of numerous downstream targets. Thus, to determine how EETs activate PI3Kα, either cardiomyocytes or hearts from wild-type mice will be perfused and treated with non-receptor activated tyrosine kinase inhibitor (Src kinase specific, SKI-606, 1μM) or a receptor tyrosine kinase inhibitor (genistein 50μM). To determine the phosphorylation status of PI3K subunits during IR, cells and tissues will be immunoprecipitated (IP) with phosphotyrosine, p85α or p110α antibodies and immunoblotted against phosphotyrosine, p85α or p110α antibodies using established techniques.

2. Experiments to identify the key mediators linking EET-mediated activation of the PI3Kα-*pm*K_{ATP} channels.

In the current study we have demonstrated that EETs render protection through a PI3Kα-*pm*K_{ATP} dependent mechanism, however, it is not clear how this occurs. To investigate this single cell contractile studies can performed by simply adding EETs or analogs of EETs and co-perfusing with either PI-103 (PI3Kα inhibitor), neomycin (binds to negatively charged PIP's and inhibits their activity) or by using a PKC blocker, chelerythrine, following hypoxic-reoxygenation injury.

3. Experiments to identify the intracellular pathways linking EET-mediated activation of the PI3Kα-*pm*K_{ATP} to the mitochondria.

To investigate how EET-mediated protection regulates Ca^{2+} overload and how this links to mitochondrial function. Changes in Ca^{2+} concentrations during and following IR injury can be assessed using fluorescent indicators in cardiomyocytes following treatment with EETs or EET analogs along with PI-103 (PI3Kα inhibitor) or GLIB (*pm*K_{ATP} inhibitor) following HR injury. Changes in the intracellular levels of Ca^{2+} can be assessed by a cytosolic specific Ca^{2+} fluorophore, Fluo-4 dye and changes in mitochondrial levels can be assessed by mitochondria specific flourophore, Rhod-2 dye.

6.4 References

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