University of Alberta

New insights into epoxyeicosatrienoic acid-mediated protective effects in cardiac cells

by

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Dedication

I dedicate this work to my lovely parents and sisters

ABSTRACT

Epoxyeicosatrienoic acids (EET) are cytochrome P450 epoxygenase metabolites of arachidonic acid. Evidence shows that they mediate protective effects in the cardiovascular system promoting cell survival. In this thesis, the major focus was to investigate if and how EETs regulate autophagy in cardiac cells during starvation. We used a dual-acting synthetic analog, UA-8 (13-(3-propylureido)tridec-8-enoic acid), possessing EET-mimetic and soluble epoxide hydrolase inhibitory properties. Our results demonstrated that UA-8 modulated an autophagic response in starved cells improving cell viability and enhancing recovery. Furthermore, UA-8 reduced both caspase-3 and total proteasome activities. Genetic as well as pharmacological inhibition of autophagy abolished the UA-8-mediated protective effects. Mechanistic studies demonstrated that sarcolemmal ATP-sensitive potassium channels might be involved in the UA-8-mediated protective effects including modulation of autophagic response. Our findings provide new evidence highlighting an important role of the autophagic response in the EET-mediated survival of cardiac cells.

CO-AUTHORSHIP

The majority of the experimental work presented in this thesis was performed by Nasser Alsaleh and Dr. Victor Samokhvalov. Many of the experiments were performed by both Nasser Alsaleh and Dr. Victor Samokhvalov in association. These are the following experiments: cell viability and recovery, caspase-3 and proteasome activities, *Atg7* silencing as well as cell culture including isolation of neonatal cardiac cells. Most of the immunoblotting experiments were performed by Nasser Alsaleh. Finally, the mitochondrial enzymatic activity as well as cardiac cells contractility experiments were performed by Dr. Victor Samokhvalov.

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LIST OF ABREVEATIONS

14,15-EEZE	14,15-epoxyeicosa-5(Z)-enoic acid
3-MA	3-methyladenine
AA	Arachidonic acid
AMC	7-amino-4-methylcoumarin
AMPK	cAMP activated protein kinase
ANOVA	Analysis of variance
Atg7	Autophagy related protein 7
cAMP	Cyclic adenosine monophosphate
CFA	Colony formation ability
COX	Cyclooxygenase
CVD	Cardiovascular disease
CVS	Cardiovascular system
CYP2J2 Tr	Mice with cardiomyocyte specific overexpression of
DCU	*N,N'-dicyclohexylurea*
DHET	Dihydroxyeicosatrienoic acid
EDHF	Endothelial-derived hyperpolarizing factor
EET	Epoxyeicosatrienoic acid
FABP	Fatty acid binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HETE	Hydroxyeicosatetraenoic acid

IHD	Ischemic heart disease
IR	Ischemia-reperfusion
IκB	I kappa B
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Ca^{2+}-sensitive K^+ channels
LC3	Microtubule-associated protein light chain 3
LDH	Lactate dehydrogenase
LOX	Lipooxygenase
LVDP	Lift ventricular developed pressure
MAPK	Mitogen-activated protein kinase
mitoK_{ATP}	Mitochondrial ATP-sensitive potassium channel
mTORC1	Mammalian target of rapamycin complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCM	neonatal cardiac myocytes
NFκB	Nuclear factor kappa B
NO	Nitric oxide
PCG1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PI3K	Phosphatidylinositide 3-kinases
PKA	Protein kinase A
pmK_{ATP}	Sarcolemmal ATP-sensitive potassium channels
PPARγ	Peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid

sEH	Soluble epoxide hydrolase
sEHi	sEH inhibitor
shRNA	short hairpin ribonucleic acid
t-AUCB	trans-4- [4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
TRPV4	Transient receptor potential vanilloid 4
UA-8	13-(3-propylureido)tridec-8-enoic acid
ULK1	UNC-51-like kinase 1

CHAPTER 1

Introduction and thesis overview

1.1 GENERAL BACKGROUND

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality worldwide. According to the American Heart Association, CVD account for 1 of every 3 deaths in the United States (56). In 2010, CVD in Canada account for 29% of all deaths with 54% of mortality due to ischemic heart diseases (IHD) (1). Therefore, CVD represent health as well as economic burdens on healthcare systems. Thus, further support toward basic as well as clinically oriented research is essential for a better understanding for identifying molecular mechanisms underlying pathogenesis of CVD, which is of great importance for development of novel therapies.

Polyunsaturated fatty acids (PUFA) are functionally important in the regulation of cellular hemostasis (145). Besides providing energy, PUFA play an important role in cell signal transduction. Arachidonic acid (AA) is an n-6 PUFA with 20 carbon atoms and four double bonds (20:4). Normally, AA is found esterified to cell membrane phospholipids. Upon a stimulus such as a hormone or a growth factor, AA is released by the action of phospholipase A_2 (PLA_2) into the cytosol. Free AA acid is rapidly metabolized to a large number of molecules known collectively as eicosanoids (145) (Figure 1.1). In addition to the well-studied and characterized AA metabolic pathways, cyclooxygenase (COX) and lipooxygenase (LOX), cytochrome *P*-450 (CYP450) enzymes also convert AA into various metabolites representing a third pathway of AA

metabolism (143). Importantly, there are two major CYP450 enzymes that metabolize AA; these are CYP450 epoxygenases and CYP450 ω-hydroxylases producing epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), respectively (144). These two major groups of AA metabolites can mediate a wide range of biological events. For instance, EETs have potent vasodilatory properties and HETEs, on the other hand, have potent vasoconstrictive properties. Describing the biological activities of these molecules is beyond the scope of this thesis and can be found elsewhere (17, 143). However, different aspects of the EET-mediated events are discussed below.

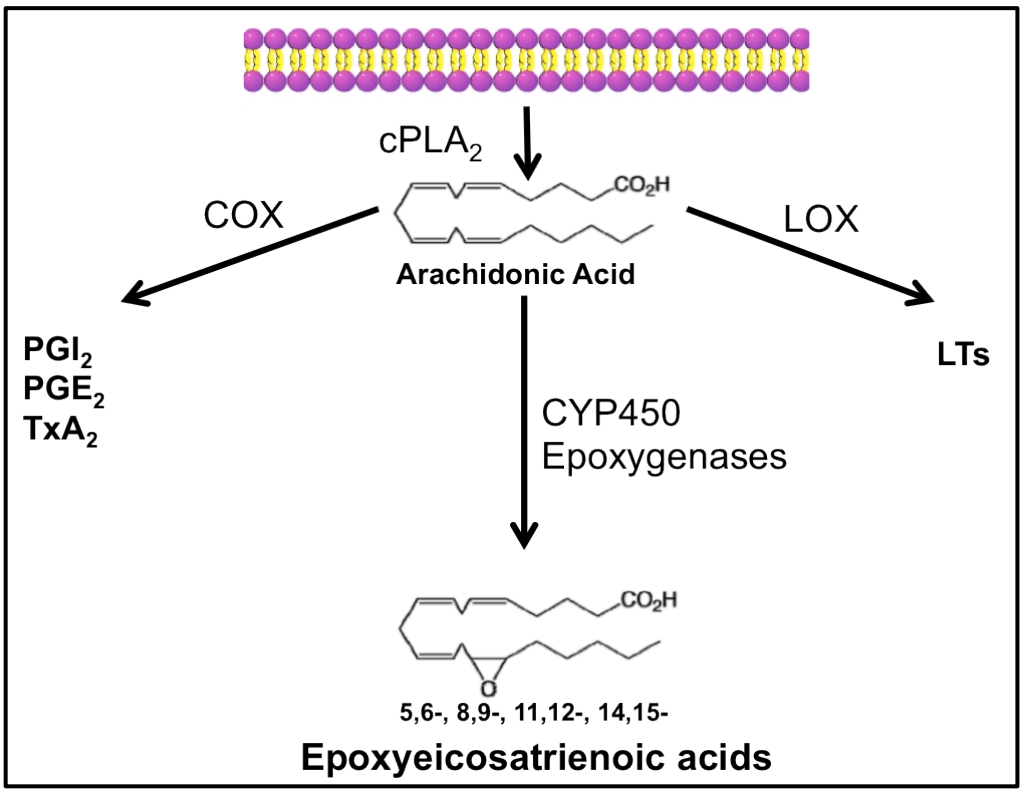

Figure 1.1 Major metabolic pathways of arachidonic acid

Esterified AA is released by the action of $cPLA_2$. Free AA is metabolized through three metabolic pathways. These are LOX, COX, and CYP450 epoxygenases converting AA to LTs, PGs, and EETs, respectively. Four EETs regioisomers are produced and each regioisomer can be produced in two configurations (enantiomers) *i.e. R/S* or *S/R*, thus a total of eight chemically distinct molecules of EETs are produced from AA.

1.2 EPOXYEICOSATRIENOIC ACIDS: AN OVERVIEW

EETs are among the most studied biologically active AA metabolites of CYP450 enzymes. They are formed by the addition of oxygen across one of the four double bonds of AA, and hence four EET regioisomers can be formed; these are 5,6-, 8,9-, 11,12- and 14,15-EETs. Furthermore, each regioisomer can be produced in two configurations (*R/S* or *S/R* enantiomers) and thus a total of eight distinct molecules can be produced by the CYP450 epoxygenases. The expression profile of CYP450 epoxygenases in different tissues determines the type and amount of regioisomer/enantiomer produced with 11,12- and 14,15-EETs among the most abundant regioisomers (82). It has been a while since scientists reported an endothelial dependent factor, other than NO and prostacyclin, to hyperpolarize smooth muscle cells; however, they could not characterize it at the time and they called it endothelial-derived hyperpolarizing factor (EDHF) (53). Most importantly, EETs were demonstrated to act as EDHFs in the cardiovascular system (CVS) (16, 49, 50, 75). This has attracted more investigators into this area of research that eventually has enhanced our understanding about the underlying molecular mechanisms of EETs (143).

Functional studies showed different cellular targets and signaling pathways for EETs. The fact that there are eight structurally distinct EET molecules, which differ in potency and/or function, has added further complexity to advancing this area of research. For example, it was found

that the R/S isomer of 11,12-EET (but not the S/R isomer) is the active isomer in dilating small renal arteries (207). As a result, some discrepancy between functional studies can be attributed to the type of regioisomer/enantiomer studied.

One of the most important aspects in studying EETs, which represents the focal point of many laboratories, is their metabolism. As EETs are chemically labile (because of their nature as epoxides), they can be degraded very readily by different enzymatic systems, mainly by soluble epoxide hydrolase (sEH). In this thesis, we will briefly introduce the CYP450 family and we will discuss the metabolism of EETs in the CVS.

1.3 CYP450 ENZYMES AND EPOXYEICOSATRIENOIC ACIDS

1.3.1 CYP450: an overview

CYP450 enzymes constitute a superfamily of membrane-bound heme-containing enzymes metabolizing different biologically active molecules such as hormones, vitamins, fatty and bile acids, drugs and environmental pollutants. CYP450 enzymes are of great clinical significance in drug metabolism as some families such as CYP3A4 and CYP2D6 metabolize a large number of drugs (161, 188). CYP450 enzymes are widely expressed and found almost in all mammalian and prokaryotic cells (170). Although liver is the primary organ of CYP450 enzymes, these enzymes occur in extra-hepatic organs such as intestine, lung and kidney (64, 135, 151). It is worth noting that the expression of CYP450 enzymes is tissue-specific (135). Thus, organ-specific toxicities can be attributed to the tissue-specific expression nature of CYP450 enzymes.

To date, 57 CYP450 genes have been identified in human (121). Among this family of enzymes, CYP3A4 is the most abundant enzyme that is accounted for metabolism of the majority of drugs (63). Genetic polymorphism of CYP450 enzymes is one of the most studied topics in drug metabolism. Inter-individual differential response to many drugs can be attributed to polymorphism of CYP450 enzymes, which is of significant consequence in regards to drugs efficacy and toxicity (93). For instance,

Mega et al reported that the platelet inhibitory effect of clopidogrel was reduced in patients who carried the CYP2C19 allele (111). Another important aspect in studying CYP450 enzymes is modulation of their activity by different factors such as diseases (25), drugs (115), nutrients (65) and genetic variation (28, 93). Of note that the change in activity of CYP450 enzymes (*i.e*. induction or inhibition) are also of great clinical importance particularly for drugs with low therapeutic index such as warfarin (4). Thus, characterizing individual enzymes is important for an optimal drug action and minimal drug interactions and adverse reactions.

1.3.2 CYP450 epoxygenases in the CVS

CYP450 enzymes were believed to be located exclusively in the liver. However, it is now well recognized that these enzymes are also found in extra-hepatic tissues such as lungs, brain, kidneys and heart (64, 135, 151). Although the liver remains to be the major organ for metabolism with predominant expression of CYP450 enzymes, extra-hepatic CYP450 enzymes play an important role in maintaining cellular homeostasis (64, 135, 151). In the CVS, several isoforms of CYP450 enzymes were identified such as CYP1A, -2A, -2B, -2C, -2E, -2J, -4A (11, 113, 142, 154), which are responsible for the metabolism of AA into different eicosanoids. The isoforms CYP1A, -2B, -2C and -2J are responsible for epoxidation of AA in humans (29, 105, 190). CYP2J and -2C are the two major families of CYP450 enzymes responsible for the synthesis of EETs in the CVS with the former being the most abundant isoform in the human heart (92, 190).

In the heart, CYP2J2 was found to be expressed in cardiomyocytes, coronary endothelial cells and smooth muscle cells (141, 146, 176, 190). Of note, reactive oxygen species (ROS) are generated normally during the process of AA epoxidation by the CYP450 epoxygenases. Interestingly, CYP2J was shown to produce very minimal amount of ROS whereas CYP2C was shown to generate a significant amount of ROS, which could be detrimental (49, 164). Therefore, CYP2J represents a better target to study the EET-mediated protective effects in the CVS. Overall, high expression profile of CYP2J2 in the heart (92) suggests that AA metabolites play an important role in cellular hemostasis.

1.3.3 CYP2J2 polymorphism in the CVS

Genetic polymorphism in CYP450 enzymes is an area of extensive investigation. Polymorphism in CYP450 enzymes can be of significant clinical importance. Considering expression of CYP450 enzymes in the CVS, polymorphism in the CYP450 enzymes is likely to cause or make persons susceptible to CVD. Because CYP2J2 is the most abundant isoform in the human heart (92, 190), scientists have investigated the association between CYP2J2 polymorphism and risk of CVD. Interestingly, it was found that polymorphism in CYP2J2 is associated with risk of myocardial infarction (107). It is of note that genetic polymorphism in CYP2J2 enzyme varies among different ethnic groups. For instance, it was found in African-Americans, but not in Caucasians, that increased risk of coronary heart disease is associated with polymorphism in the CYP2J2

epoxygenase (G-50T) (94, 166). These and other studies (100, 138, 166) provide evidence for the clinical importance of CYP2J polymorphism. Although evidence is accumulating for the association between CYP450 polymorphism and CVD, other studies (48, 203) showed that there was no association between CYP2J polymorphism and ischemic heart disease. Discrepancy between these studies can be attributed to different factors such as genetic background of the studied populations. Therefore, further research is warranted to better understand the association between CYP2J2 polymorphism and the risk of CVD.

1.4 MECHANISMS OF ACTION OF EPOXYEICOSATRIENOIC ACIDS

EETs mediate a broad spectrum of biological effects including vasodilation (139, 140), suppression of inflammation (15, 34, 127), attenuation of apoptosis (12, 23, 35) and prevention of platelet aggregation (12). EETs are capable of modulating many different cell signal transduction pathways (143, 165). There has been controversy regarding the exact mechanism(s) of action for the EETs. In the past two decades, EETs were shown to act through several mechanisms (163). However, the interrelation between these mechanisms is still poorly defined (125, 143, 165).

Evidence is emerging to support the notion that EETs mediate their effects through a membrane receptor. For instance, using a derivative of 14,15-EETs that was tethered with silica beads (to prevent it from entering the cell), Snyder et al demonstrated that EETs were still able to inhibit dibutyryl cAMP-induced aromatase activity (159) suggesting that EETs work through a membrane receptor. Furthermore, in an attempt to identify a putative receptor, Wong et al identified a population of specific binding sites in U-937 cells, a monocyte cell line, that bind EETs with high affinity, which is believed to be protein in nature as the binding was sensitive to proteases (186). The authors found that the binding was decreased in the presence of cholera toxin and dibutyryl cAMP, which was reversed by the use of protein kinase A inhibitor. Accordingly, the authors proposed that

EETs bind to a membrane receptor, which leads to an increase in cAMP levels and activation of PKA. Additionally, evidence supporting the existence of EET receptor stems from several other studies that demonstrate activation of several signaling pathways such as PI3K/Akt, which are typically activated by membrane receptors (180).

Besides being proposed to act through a membrane receptor, EETs have been shown to act as intracellular signaling molecules and to bind to intracellular signal transduction elements (143). For instance, EETs were found to bind to peroxisome proliferator-activated receptor gamma subtype producing their anti-inflammatory effect (101) and K_{ATP} channels resulting in a reduction of their sensitivity to ATP (102). Furthermore, the notion that EETs act as intracellular signaling molecules is also supported by the findings that EETs bind fatty acid binding proteins (FABP) (183, 184). Moreover, the fact that EETs are synthesized from free AA within the cell or are released (by the action of PLA_2) from stored EETs in the plasma membrane supports the intracellular mechanism. All together, these studies provide evidence that EETs may act as intracellular signaling molecules.

1.5 BIOLOGICAL FUNCTIONS OF EPOXYEICOSATRIENOIC ACIDS IN THE CARDIOVASCULAR SYSTEM

1.5.1 EETs and relaxation of the vasculature

Following the identification of EETs as EDHF in the mid 1990 (16, 50, 75), remarkable attention was given to study these molecules. There is a good understanding of the mechanisms of action underlying the EET-mediated vasodilatory effect, which was illustrated in *in-vivo* and *in-vitro* experimental models (143). Interestingly, it was found that the vasodilatory effect of EETs occurs through multiple mechanisms. For instance, it was found that EETs open Ca^{2+}-activated K^+ (K_{Ca}) channels (EDHF effect) causing hyperpolarization of smooth muscle cells (16, 37, 55, 73, 80). Evidence suggests that they directly activate reconstituted K_{Ca} channels (37). It is of note that the EET-mediated vasorelaxation through K_{Ca} channels occurs preferentially in small coronary arterioles rather than larger coronary arteries suggesting that they regulate the coronary circulation (129). Another mechanism for the EET-mediated vasodilation effect is the activation of transient receptor potential vanilloid 4 (TRPV4) channels (38, 181). Earley et al found that suppression of TRPV4 in intact cerebral arteries abolished the vasodilatation effect of EETs (38). Taken together, numerous independent laboratories have studied the EET-mediated vasodilatory properties and particularly as EDHF (16, 55, 73).

These studies suggest that the EET-mediated vasorelaxation occurs through multiple mechanims.

1.5.2 EETs and ischemia/reperfusion injury

Accumulating evidence suggests that EETs are protective against the ischemia/reperfusion (IR) injury (82, 125, 155). EETs were found to produce protective effects through multiple mechanisms (189). Our group showed using transgenic mice with cardiomyocyte-specific overexpression of CYP2J2 (*CYP2J2* Tr) that these mice have improved left ventricular developed pressure (LVDP) after 20 min of ischemia and 40 min of reperfusion compared to WT mice (152). Similarly, we demonstrated in the same study that administration of exogenous EETs produce parallel results. Furthermore, using pharmacological inhibitors, we demonstrated that EET-mediated protective effects involved activation of mitoK_{ATP} channels and the p42/p44 MAPK pro-survival signaling pathway. In another study we showed that hearts from sEH null mice had improved LVDP and reduced infarction following IR injury (154). Using the EETs putative inhibitor 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), we demonstrated that these effects are EET-dependent. Moreover, our mechanistic studies showed that EET-induced effects were mediated through the PI3K signaling pathway as well as sarcolemmal/mitochondrial K_{ATP} channels (154). Others studies (62, 126) also confirmed the involvement of K_{ATP} channels in the EET-mediated effects. In addition to the mentioned mechanisms for the EET-mediated myocardial protection

against the IR injury, other studies showed other cellular targets including opioid receptors (60) and the signal transducer and activator of transcription–3 (STAT3) signaling pathway (112).

Besides using genetic approaches and administration of exogenous EETs, there have been efforts to synthesize EET analogues and soluble epoxide hydrolase inhibitors (sEHi) (83). In the past decade or so different classes of sEHi were introduced (84) (discussed later in this chapter). Furthermore, dual acting surrogates, which possess both EET-mimetic activity and sEH inhibitory properties, have been synthesized (43). Our group demonstrated using a synthetic analogue that possesses EET mimetic and sEH inhibitory properties named 13-(3-propylureido)tridec-8-enoic acid (UA-8) that it improved LVDP and reduced the size of infarction following IR injury (8). In the studies presented in this thesis we used UA-8 in most of the experiments. To confirm that our findings are EET-dependent, we used, in key experiments, 14,15-EET as a model EET molecule.

1.5.3 EETs and inflammation

Another important biological function of EETs is their anti-inflammatory properties. It was found that EETs suppress the inflammatory response through direct inhibition of transcription factor nuclear factor kappa B (NFκB) and I kappa B (IκB) kinase (127). EETs were also found to inhibit the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 and E-selectin,

through a mechanism that is independent of EET-mediated opening of K_{Ca} channels (127). Interestingly, it was demonstrated that not all EET regioisomers have the same anti-inflammatory effects. They found that 11,12-EET inhibited TNFα-induced VCAM-1 expression the most, followed by 8,9- and 5,6-EETs. Surprisingly, 14,15-EET did not inhibit TNFα-induced VCAM-1 expression. This could be a source of discrepancy between functional studies and therefore should be considered when comparing findings of independent laboratories. Recently, using three different genetically modified mice (CYP2J2 and -2C8 transgenic and sEH KO), which all express high levels of EETs, Deng et al demonstrated in *in-vivo* and *in-vitro* systems that these mice had a reduced activation of NFκB in response to endotoxin (33). To confirm these results, the authors used the putative EET antagonist, 14,15-EEZE, and CYP450 epoxygenase inhibitor and found that both agents abolished the EET-mediated effects. All together, these results show that EETs mediate their anti-inflammatory properties through different mechanisms suggesting that EETs are capable of modulating different intracellular signal transduction pathways.

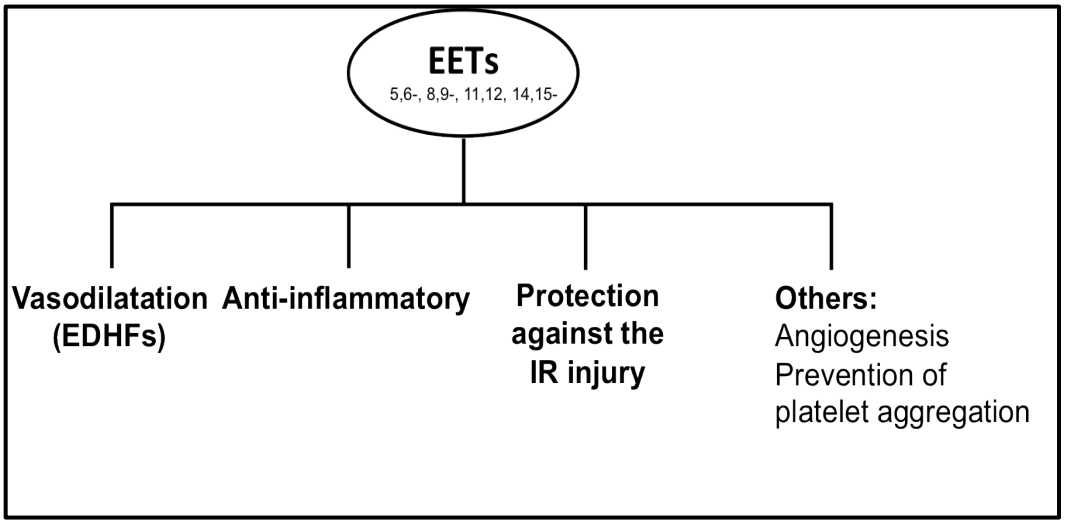

Figure 1.2 EET-mediated biological functions in the cardiovascular system (adapted from (165))

EETs mediate various functions in the CVS. They regulate the vasculature tone (as EDHF). EETs modulate the inflammatory response, protect against IR injury and can mediate other functions such as angiogenesis and prevention of platelet aggregation.

1.6 DEGRADATION OF EPOXYEICOSATRIENOIC ACIDS

1.6.1 The soluble epoxide hydrolase pathway

Epoxide hydrolases (EH) catalyze the hydrolysis of epoxides, by adding water, to form diol derivatives (128). EHs are members of the α/β hydrolyse fold family (117). In mammals, several EH exist. However, soluble (sEH) and microsomal (mEH) epoxide hydrolases are the most studied ones. These enzymes are expressed widely in the different body tissues (118). They function to detoxify and catabolize xenobiotics as well as regulate cell signaling elements (118). Besides their distinct subcellular localization, evidence indicates an important role for sEH in the CVS (30, 158, 201). sEH is highly expressed in the heart (41). Thus, it is reasonable to predict their implication in the metabolism of EETs. Indeed, it was found that inhibition of sEH represents an important strategy for maintaining the beneficial biological effects of EETs for longer time (84, 158, 201, 206). Genetic as well as pharmacologic inhibition of sEH has been shown to effectively reproduce the EET-mediated biological effects (155).

Different generations of sEHi were synthesized and tested for their protective effects in the CVS (84). The first class of sEHi was epoxide-containing compounds. They had *in-vitro* transient inhibitory effect; they were ineffective *in-vivo* (118). Later, other classes of sEHi were introduced such as ureas, amides and carbamates (118). 1,3-disubstituted urea

derivatives were more potent at inhibiting sEH, and they were effective in several *in-vitro* and *in-vivo* experimental models (118).

EETs are chemically unstable and can be rapidly metabolized by sEH (encoded by *EPXH2)* to the biologically less active diol derivatives, dihydroxyeicosatrienoic acids (DHETs) (19, 202). Numerous studies investigated sEHi as a therapeutic strategy in exploiting the EETs beneficial cardiovascular properties. For instance, it was shown that treating spontaneously hypertensive rats with the selective sEH inhibitor, *N,N'-dicyclohexylurea* (DCU), reduces blood pressure (201). These findings were confirmed using a genetic approach. It was found that mice with disrupted *EPXH2* gene had lower blood pressure suggesting sEH as a novel therapeutic target for hypertension (158). We and others have tested a number of sEHi using different experimental models. For instance, we found that treatment of C57BL6 mice with trans-4- [4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) was protective against IR injury (20). Furthermore, t-AUCB treatment significantly improved post-ischemic LVDP and reduced the size of infraction. All together, these findings and the advantage that sEHi produce minimal adverse effects (84) make sEHi potential candidates in the treatment of cardiovascular disease.

1.6.2 Non-sEH pathways

Despite the fact that sEH is the main pathway for EETs degradation, a number of other pathways were shown to contribute to the

degradation of EETs (201) (Figure 1.3). For instance, it was found that EETs undergo β-oxidation and chain elongation as well as reincorporate into the sn-2 position of cell membrane phospholipids (165). Interestingly, sEH is not always the main metabolic pathway. It was shown in human skin fibroblasts (44) and human coronary artery endothelial cells (46) that β-oxidation is the predominant pathway. Of note, inhibiting sEH was found to enhance the degradation of EETs through the other metabolic pathways (45). In addition to the previously mentioned pathways for EETs degradation, EETs can be metabolized by cyclooxygenase (18, 77) and conjugated to glutathione (162). Another important consideration is the binding of EETs to FABP. Widstrom et al showed that EETs bind FABP with a relatively high affinity, which may serve as storage pools of EETs (183). Interestingly, the same group demonstrated later that binding of EETs to FABP might represent a way to avoid sEH-mediated degradation of EETs (184). Taken together, this area of research is very important as a better understanding of the EETs metabolism and corresponding EETs metabolites could help in designing novel sEHi as well as identifying novel active metabolites.

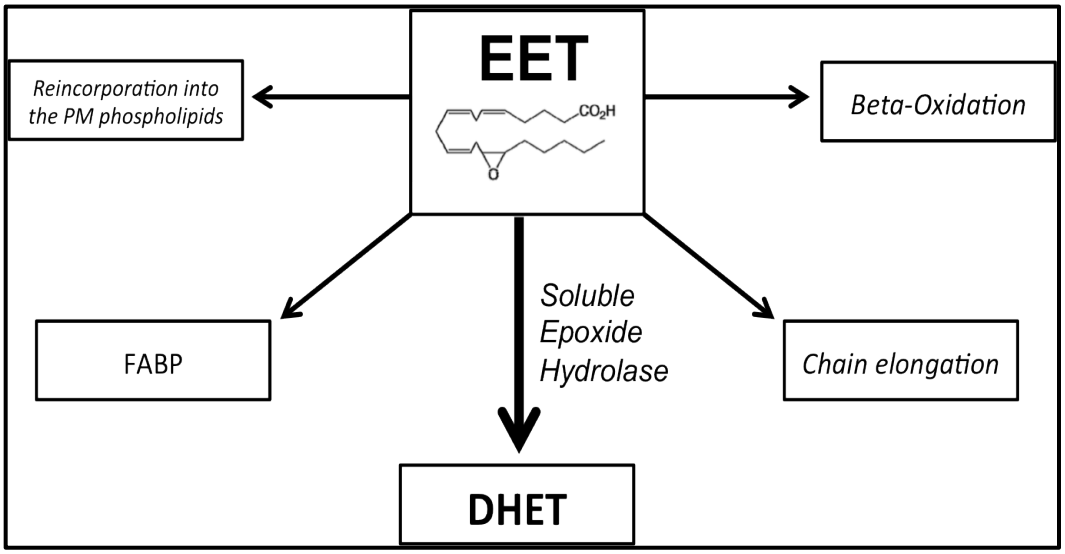

Figure 1.3 Major metabolic pathways of the EETs (adapted from (165))

EETs undergo different metabolic pathways. They are mainly metabolized by the sEH to form the corresponding diol derivatives, DHET. Other metabolic pathways include beta-oxidation, chain elongation and reincorporation into the plasma membrane phospholipids. Of note, EETs can bind fatty acid binding proteins, which may serve as a storage pool for EETs.

1.7 AUTOPHAGY AND CARDIOPROTECTION

1.7.1 Autophagy: an overview

Autophagy is an evolutionarily conserved, highly regulated process where intracellular macromolecules and organelles are sequestered into a vacuole known as autophagosome (90, 197, 198). Eventually, an autophagosome fuses with a lysosome and the contents are degraded (figure 1.4). Autophagy is an essential cellular process for the turnover of aged and dysfunctional proteins and organelles functioning as a cellular quality control system (90, 197, 198). It is a complex dynamic process (the autophagic flux) that is regulated by proteins known as autophagy related (Atg) proteins as well as class III PI3K. This dynamic process involves initiation and formation of phagophore (isolation membrane), nucleation, expansion, degradation of content and recycling of materials. About 30 Atg proteins have been identified in yeast, many of which have been also identified in mammalian cells (199). A number of theses proteins such as Atg3, Atg7 and Atg8 (LC3, the mammalian homolog) mediate ubiquitylation-like modifications (171). LC3 is believed to play a role during elongation of phagophore and fusion of completed autophagosome. (199). Recent research has revealed a large body of information regarding the molecular mechanisms controlling this complex biological process. Interestingly, alteration in the level of autophagy has been found to underlie pathogenesis of neurodegenerative disorders such as Alzheimer's and Huntington's disease as well as cancer among others

(147). Paradoxically, studies show that both the upregulation and downregulation of autophagy may be therapeutically beneficial (147). For instance, it was found that induction of autophagy suppresses tumor formation (98). In contrast, suppression of autophagy found to be beneficial to anticancer therapy (3, 5). Although it is currently unclear how can both the induction and inhibition of autophagy be therapeutically useful, these findings provide evidence that autophagy represents an important potential target for therapeutic intervention.

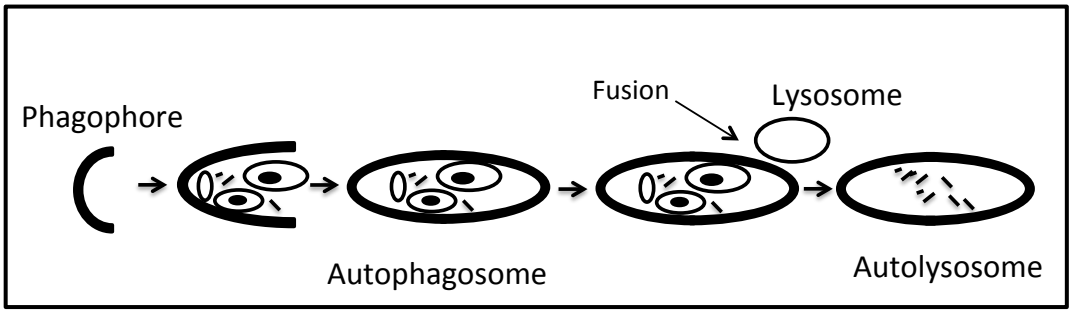

Figure 1.4 The process of autophagy (adapted from K Nishida et al, 2008 (123)).

Atg regulate autophagy and participate in the formation of an autophagosome, which eventually fuses with a lysosome to form autolysosome and the contents are degraded. Autophagy functions as a quality control system where damaged organelles and aged proteins are turned over and utilized as a source for energy and recycling of building blocks.

1.7.2 The crosstalk between autophagy and apoptosis

The molecular link between autophagy (type II cell death) and apoptosis (type I cell death) remains unclear. Although autophagy is considered as a type of cell death, substantial body of evidence suggests that autophagy plays an important role in cell survival in the different body tissues (36, 72, 136). This has been demonstrated in various *in-vitro* and *in-vivo* experimental models in health and disease (157). The interplay between autophagy and apoptosis is complex at the molecular level, and some proteins have been shown to regulate both processes such as the B cell lymphoma 2 (BCL-2) family. Numerous studies demonstrated the involvement of apoptotic regulators in autophagy. For instance, Pattingre et al found that the anti-apoptotic protein BCL-2 inhibits autophagy through its interaction with beclin 1 (134). The authors proposed that autophagy is essential to adapt to cellular stress and that increased autophagy beyond physiological levels can lead to cell death. In an elegant study, Shimizu et al showed that Bax/Bak$^{-/-}$ mouse embryonic fibroblasts undergo autophagic cell death in response to several apoptotic stimuli and that autophagic inhibitors suppress cell death (156). The inhibition of caspase-8 was also found to trigger autophagy through Atg7 and beclin 1 (200). On the other hand, other studies also demonstrated that disruption in autophagy affects apoptotic cell death. Both genetic as well as pharmacologic inhibition of autophagy triggers apoptosis (13). Gonzalez-Polo et al found that amino acid starved lysosome-associated membrane

protein 2 (LAMP-2) negative cells had accumulated autophagic vacuoles, which were followed by cell death with apoptotic hallmarks (58). Taken together, evidence is emerging for crosstalk between autophagy and apoptosis, yet the underlying molecular interaction is still to be elucidated.

1.7.3 Autophagy in the cardiovascular system

Whether autophagy contributes to cell survival or death in the CVS have long been debated. Several lines of evidence support the notion that autophagy works as a protective mechanism in the sense that (1) aged proteins and organelles need to be turned over for optimal functioning, and (2) autophagy maintains energy demands under compromised conditions such as starvation. However, it has also been proposed that exceeding physiological levels of autophagy may lead to execute cell death (31).

A classical example for the role of autophagy in the CVS is the genetic deficiency of LAMP-2, a structural protein necessary for fusion of an autophagosome with a lysosome, which results in Danon's disease and is manifested as cardiomyopathy (124). Accumulation of autophagic vacuoles is a hallmark of Danon's disease (124). Heart failure is a major cause for morbidity and mortality (1, 56). The molecular mechanisms responsible for the development of heart failure are still poorly understood. Considering autophagy is an essential process for cell survival, it is reasonable to hypothesize that dysfunction in the autophagic machinery results, or at least, contributes to pathogenesis of heart failure. In fact, scientists have studied this hypothesis and found that indeed a

dysfunction in the autophagic flux can results in cardiomyopathies. For instance, Nakai et al investigated the effect of silencing *Atg5* encoding an essential autophagic protein, in the normal and failing heart (120). Interestingly, the authors found that the *Atg5* silenced mice developed cardiac hypertrophy, left ventricular dilatation and contractile dysfunction associated with morphological changes of the sarcomere and mitochondria (120). Furthermore, they also found these mice are more susceptible to β-adrenergic and pressure overload-induced stress. These findings demonstrate that autophagy is necessary at basal levels and is important for the CVS homeostasis. In the contrary, other studies found that increased autophagy underlies heart failure. For instance, Knaapen et al reported that cardiomyocytes in failing hearts exhibited autophagic cell death (91). It is still controversial whether autophagy enhances or suppresses heart failure and thus further studies are warranted to elucidate the exact underlying molecular mechanisms.

1.7.4 Autophagy and ischemia/reperfusion injury

It has been long time since Sybers et al showed the formation of autophagosomes after transient deprivation of oxygen and glucose in fetal mice heart organ culture (169). Recent work demonstrates that autophagy is robustly induced following IR injury in different experimental models (40, 71, 110, 175, 191). The functional role of autophagy during IR injury has yet to be fully understood. Numerous studies have shown that induction of autophagy contributes to cell death. For instance, it was found that a

decrease in the expression level of beclin 1 was associated with increased survival in neonatal and adult cardiomyocytes following IR injury (175). Furthermore, Matsui et al reported that the reperfusion injury was significantly attenuated in beclin $1^{+/-}$ mice (110). Other studies, however, showed contradictory findings. It was found that induction of autophagy enhances cell survival. For instance, more than three decades ago, Decker et al found following 20 or 40 min of ischemia that recovered cardiomyocytes had a robust increase in autophagy (32). Another study also showed that autophagy enhanced protection against IR injury (70). In this study, the authors reported that downregulation of autophagy by dominant negative mutant of Atg5 deteriorated the cellular injury. Another line of evidence supporting the notion that autophagy is protective rather than detrimental to the myocardium stems from a study demonstrating that IPC increased the formation of autophagy and that inhibition of autophagy abolished the ischemic preconditioning (IPC)-induced protective effects (68). These findings suggest that autophagy is important for cell survival and salvage of the myocardium. All together, considering the role of autophagy in cell survival, it is plausible that activation of autophagy at early stages contributes to cell survival, however, over-activation or activation of autophagy beyond certain limits contributes to cell death.

It is well established that autophagy can be induced by conditions such as nutrient deprivation (starvation), hypoxia and ROS (90, 123). These and other elements are important components of the metabolic

changes during IR injury (194). Nutrient deprivation is a strong activator of autophagy and thus starvation represents a good model for studying autophagy (90, 197, 198). Starvation is a unique model where autophagy and apoptosis occurs simultaneously (108). Our knowledge about the EET-mediated regulation of cell death is limited to their anti-apoptotic properties (12, 23, 35). Therefore, we sought to study autophagy as a component of the EET-mediated protection in cardiac cells during cellular starvation.

1.8 THESIS OVERVIEW

1.8.1 Rationale

The biological effects of EETs in the CVS such as vasodilatation (139, 140), suppression of inflammation (15, 34, 127) and protection against IR injury (125) are well documented. Nevertheless, the underlying molecular mechanisms remain unclear. Our group is interested in defining the molecular mechanisms of the EET-mediated cardioprotection following IR injury. We previously demonstrated that EET-mediated cardioprotection against IR injury occurs through multiple mechanisms (125) such as activation of p42/p44 MAPK signaling pathway and K_{ATP} channels (154).

Evidence shows that EETs are also involved in the regulation of cell death pathways (12, 23, 35); however, our knowledge is limited to their anti-apoptotic properties. Autophagy is a highly regulated process where intracellular macromolecules are sequestered into double membrane vesicles for materials to be degraded and recycled. Although autophagy may be classified as a type cell death, accumulating evidence suggests cells trigger an autophagic response to starvation-induced stress as a protective survival mechanism (108). This is particularly important in terminally differentiated cells such as cardiac cells. Indeed, evidence suggests that activation of autophagy favor cell survival in cardiac cells (36). Although it is not yet clear how autophagy works as a cell death pathway and at the same time promotes cell survival, there is a general

consensus that the extent of the autophagic response is what determines the role of autophagy in cell death or survival.

Importantly, it was reported that inhibition of autophagy led to loss of IPC-mediated cardioprotection against IR injury (68). EETs were shown to activate pro-survival pathways similar to those mediated by IPC (82, 143). Therefore it is reasonable to suggest that EETs might be involved in the regulation of autophagy promoting cell survival. To date, there is no documentation regarding the regulation of autophagy by EETs. Thus, we sought to investigate if and how EETs regulate the autophagic response in cardiac cell during starvation.

1.8.2 Hypothesis

Increased levels of EETs in amino acid starved HL-1 cells and neonatal cardiomyocytes modulates an autophagic response promoting cell survival and attenuating starvation-induced cellular death.

1.8.3 Objectives

1. To investigate whether EETs protect cells from cellular death triggered by amino acid starvation in HL-1 cells and neonatal cardiomyocytes.
2. To investigate if EETs modulate an autophagic response during amino acid starvation in HL-1 cells and neonatal cardiomyocytes, and whether it is necessary for mediating the protective effects of EETs.

3. To assess the involvement of *pm*K_{ATP} channels in the EET-mediated protective effects and EET-mediated modulation of an autophagic response during amino acid starvation in HL-1 cells and neonatal cardiomyocytes.

1.8.4 Significance

Cardiovascular diseases represent a major cause for morbidity and mortality worldwide. In Canada, 54% of mortality is attributed to ischemic heart diseases (1). Thus, elucidating the molecular mechanisms underlying myocardial IR injury is of great clinical importance. Although promising results of several pharmacological agents such as adenosine have been demonstrated in experimental studies (194), these results were limited or could not be translated into the clinical settings. This can be attributed to several reasons such as experimental animals are young and healthy whereas people with ischemic heart diseases are usually elder with comorbidities (194). Our ultimate goal is to better understand the EET-mediated molecular events in an attempt to find novel potential therapeutic targets for attenuating the IR–induced myocardial injury.

CHAPTER 2

Epoxyeicosatrienoic acids protect cardiac cells during starvation by modulating an autophagic response*

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2.1. INTRODUCTION

Cell turnover and maintenance of cellular homeostasis are tightly regulated processes, which balance the demand to remove damaged cells and prevent widespread effects. Cells respond to stress by activating a variety of pathways enabling them to sense changes in their environment, such as starvation, hypoxia and mechanical damage. Dependent upon the extent and nature of the stressor, cells initiate responses that can promote either survival or death pathways eliminating damaged cells. The molecular switches between these opposite responses involve a complex array of signals and adaptive pathways determining whether the cell will survive or die.

Arachidonic acid is a polyunsaturated fatty acid normally found esterified in the plasma membrane. It can be released in response to several stimuli including ischemia and stress (97, 143, 145). Free AA can be metabolized by cytochrome P450 epoxygenases to EET, which are further metabolized to dihydroxyeicosatrienoic acids via sEH or incorporated into membranes (45, 88). EETs are lipid mediators that act as potent cellular signaling molecules regulating key cellular processes, such as limiting mitochondrial damage, inhibiting apoptosis and reducing inflammatory responses (35, 59, 87, 127). Despite extensive research efforts investigating the biological effects of EETs, their intrinsic mechanism(s) of action remains poorly understood (82). While there is no known EET receptor, evidence suggests that they act as intracellular

signaling molecules affecting proteins such as cardiac ATP-sensitive potassium channels (*pm*K_{ATP}) (7, 95, 103). Moreover, EET-mediated signaling has a role in cancer progression by stimulating cell proliferation, survival, migration and invasion (130).

Depending upon the extent of the cellular stress and specific survival mechanism(s) activated, a predominance of one pathway such as, autophagy over another, such as apoptosis, will determine the fate of the cell. Autophagy represents an evolutionarily conserved catabolic process in which intracellular macromolecules and organelles are sequestered in autophagosomes for recycling (24). Autophagy plays an essential role in cellular response to stress and is an important survival mechanism of terminally differentiated cells such as cardiomyocytes (81, 172, 185, 195). It has been suggested that resistance of cells to environmental stress factors, including starvation, vastly depends on their ability to activate compensatory reactions providing rapid turnover of damaged molecules and organelles such as mitochondria (96, 177). Preservation of mitochondrial integrity by autophagy represents a biologically beneficial strategy as preserved mitochondria can greatly contribute to prolonging cell survival (57, 167). Stressed cells solely rely on the coordination of multiple response pathways that are controlled at the molecular level by a number of highly conserved molecules, such as AMP-activated protein kinase (AMPK). AMPK acts as an intracellular sensor of energy status, which is activated by an increase in the intracellular AMP:ATP ratio,

including response to metabolic stress observed in starvation (179). Once activated, AMPK switches on catabolic pathways that generate ATP, while switching off ATP-consuming processes, such as cell growth and proliferation, and activating autophagy (85). Other critical molecules like *pm*K_{ATP} channels are involved in the cellular response by regulating ionic homeostasis under conditions of metabolic stress; while these channels have demonstrated cardioprotective effects their role in regulating cell death pathways is limited (168). Excessive injury of cardiomyocytes in the heart results in collapse of cardiac function. Therefore, unraveling the mechanisms, which regulate the balance between autophagic-mediated cellular survival and apoptosis-associated cell death, will further our understanding of the cardiovascular system. Our understanding of EET involvement in regulating cell death and survival pathways is limited to their anti-apoptotic effect; moreover, nothing is known regarding EET regulation of autophagy (196). Modulating cellular death mechanisms, such as autophagy, by EETs is an under investigated aspect of cardiovascular biology that can provide new insight. In order to address this aspect, we examined protective effects of EETs on starved cardiac cells. In this study, we demonstrated that EETs modulate the autophagic response in starved cardiac cells through mechanisms involving activation of *pm*K_{ATP} channels and AMPK.

Consequently, the EET-mediated response protected mitochondrial function, which resulted in a healthier mitochondrial pool and increased viability of the starved cardiac cells. Thus, we report a novel EET-mediated protective mechanism for cardiac cell survival during starvation.

2.2. MATERIALS AND METHODS

2.2.1 Cell cultures

HL-1 cardiac cells (mouse atrial cardiomyocyte tumor lineage) were a kind gift from Dr. Claycomb (New Orleans, USA). Cells were cultivated in Claycomb media supplemented with glutamine and norephinephrine as described previously (26). HL-1 cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Neonatal cardiomyocytes were isolated from 2-3 days old rat pups as described (150). Isolated cardiomyocytes were cultivated in DMEM media with 10% FBS at 37°C in humidified incubator maintaining 5% CO2 and 95% air. Cell viability was assessed by using Trypan blue exclusion as described earlier (174). MTT assay was employed to estimate the total cellular metabolic activity based on the reduction of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) by mitochondrial dehydrogenases (178). Activity of LDH released from injured cells was measured in cultivation medium based on conversion of MTT into formazan as described (2) Beating rate was estimated by counting the number of beats per minute in 5 different cell clusters in 5 independently blinded experiments.

2.2.2 Treatment protocols

Starvation was modulated by incubating cells in amino acid and serum-free buffer as described (14). In this study, we utilized a novel EET-analogue, UA-8 (13-(3-propylureido)tridec-8-enoic acid (1μM)) that possesses EET-mimetic and sEH inhibitory properties (8). In order to

block EET-mediated effects, we utilized the putative antagonist, 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE, 10μM). Control experiments utilized 14,15-EET (1μM) as an EET model.

2.2.3 Colony formation assay

Colony formation ability (CFA) was performed as described previously (104). Briefly, HL-1 cells were treated and starved for 24 h, after which floating cells were harvested and plated (1000 cells/1 cm^2) into normal drug-free Claycomb media for 72 h. Cells were stained with 1% crystal violet for 30s after fixation with 4% paraformaldehyde for 5 minutes. The number of colonies formed, defined as >50 cells/colony, were counted.

2.2.4 Inhibition of autophagy

Silencing of Atg7 expression was achieved by transfection of HL-1 cells with plasmids expressing short hairpin RNA (shRNA) against the mouse Atg7 gene (OriGene Technologies, Rockville, MD). Atg7 targeted shRNA and scrambled negative control were cloned into a pGFP-V-RS plasmid under a U6 promotor. Plasmids were amplified in the K-12 strain of *Escherichia coli* then purified using the EndoFree plasmid purification kit (Qiagen, Valencia, CA). Cells were transfected with Lipofectamine 2000 in accordance to manufacturer's instruction. Transfection efficiency with shRNA plasmids was determined qualitatively by the expression of green fluorescent protein (GFP). Cells were subjected to starvation 24 h after transfection, and the knockdown efficiency of the plasmids was assessed

by immunoblotting. Control experiments were performed where 3-methyladenine (Sigma-Aldrich) was dissolved in DMSO and added to cardiac cells (5 mM) for 24 h to inhibit autophagy.

2.2.5 Western blot assay and antibodies

HL-1 cells or neonatal cardiomyocytes were treated as described above, washed with ice-cold phosphate buffer saline (PBS) and harvested at different time points (0, 12, 24, 36 and 48 h) using ice-cold lysis buffer (20 mM Tris-HCl, 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 M sucrose, 1 mM DTT, 1% triton-X100 and protease/phosphatase inhibitors). Cell lysates were incubated on ice for 10 min, then centrifuged at 13,000*g* for 15 min (4°C). The Bradford assay was used to measure total protein content in supernatants. 20 μg of protein was resolved in 15% SDS-polyacrylamide gel and then transferred electrophoretically to polyvinylidene fluoride membranes, which were then blocked with 5% non-fat milk in TBS-T buffer (0.15 M NaCl, 3 mM KCl, 25 mM tris hydroxymethyl methylamine and 0.1% tween-25, pH 7.4) for 1 h at room temperature. Membranes were washed three times with TBS-T buffer and then incubated overnight at 4°C with anti-LC3 antibody (Cell Signaling) to detect both LC3-I and LC3-II. Membranes were washed as described above and incubated with horseradish-peroxidase linked anti-rabbit IgG secondary antibody (Invitrogen) for 2 h at room temperature followed by washing as described above. Other antibodies utilized included AMPKα (Cell Signaling), Phospho-AMPKα (Thr172) (Cell Signaling), VDAC1

(Abcam), SDH-A (Cell Signaling), COX IV (Cell Signaling), β-actin (Cell Signaling), or GAPDH (Cell Signaling) antibodies. Chemiluminscence substrate reagents were used to detect signals. Relative band intensity to control was measured using Image J software (NIH, USA). Immunocytochemistry (ICC) was used to detect autophagosomes using LC3 antibody (Cell Signaling) according to manufacturer's instructions.

2.2.6 Assessment of mitochondrial respiratory chain enzymatic activities

Citrate synthase (CS), succinate dehydrogenase (SDH), and cytochrome c oxidase (COX) were assayed spectrophotometrically in cell lysates as previously described (167). Assessments were repeated in 3 independent experiments and enzymatic activities were expressed as nmol/min/mg protein.

2.2.7 Electron microscopy

HL-1 cells were grown on glass bottom dishes (MatTek, Ashland, MA, USA) and undergone starvation treatment as described above for 24 hours. Cells were then rinsed with PBS and fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate for 30 minutes. Cell monolayer was then post-fixed in 1% sodium tetroxide in 0.1 M sodium cacodylate for 30 minutes on ice and in the dark. 2% uranyl acetate was used for en-block staining of the samples for 30 minutes on ice and in the dark. Dehydration was done by increasing concentrations of ethanol (50-100%). Finally, resin-filled beams were transferred upside-

down on top of the cells and left at 60c incubator for 48 hours to polymerize. Imaging was done by a Philips 410 electron microscope, using Megaview III soft imaging system and iTEM software. Experiments were repeated 3 independent times.

2.2.8 Caspase-3 and 20S proteasome activity assays

Caspase-3 activity was assessed using a spectrofluorometric assay as described previously (153). 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). Total proteasome activity assay was determined in cytosolic fractions monitoring the release of AMC by proteolytic cleavage of the peptide Suc-LLVY-AMC (CHEMICON, Inc. USA) by 20S proteasomes. Fluorescence was monitored in both caspase-3 and total proteasome assays at wavelengths of 380 nm (excitation) and 460 nm (emission). Specific activities were determined from a standard curve established with AMC**.**

2.2.9 Statistical analysis

Results are presented as means ± S.E.M. Statistical analysis used ANOVA with a Bonferonni post hoc test; P <0.05 was considered statistically significant.

2.3. RESULTS

2.3.1 UA-8 preserved viability and functional activity of HL-1 cardiac cells during starvation

The protective effect of UA-8 was evaluated by using Trypan blue exclusion, which reflects loss of cell membrane integrity and cell death. Figure 2.1A demonstrates the dynamics of cell death during starvation. Starvation induced significant cell death in control groups, which progressively increased over time. After 48 h, more than 75% of control cells were dead. Protection of cell viability conferred by UA-8 was observed for up to 48 h of starvation. By contrast, co-treatment with the putative EET inhibitor, 14,15-EEZE, abolished the protective effects of UA-8, while 14,15-EEZE treatment alone had an even greater rate of cell death compared to the control.

We also employed an alternative test of cell viability based on accumulation of the reduced form of MTT in mitochondria, which reflects the ability of cells to maintain oxidative metabolic activity (178). Starvation induced a robust accumulation of formazan in HL-1 cells within 24 h in all experimental groups, except UA-8, suggesting a rapid activation of mitochondrial metabolic activity was initiated to provide energy for cell survival in response to starvation (Figure 2.1B). The initial activation subsided with a dramatic decline in cellular metabolism. Treatment with UA-8 significantly delayed the metabolic collapse of starved HL-1 cells. Co-treatment with 14,15-EEZE abolished the protective effect of UA-8.

The ability of cells to recover from stress and form new colonies is an evolutionary mechanism involved in survival and expansion. We measured the ability of HL-1 cells to form colonies after 24 h of starvation by employing a crystal violet-based test. We observed that only 15% of cells derived from control groups were able to recover and form colonies, while 35% of UA-8 treated HL-1 cells were able to recover (Figure 2.2A). The protective effect of UA-8 was abolished by co-treatment with 14,15-EEZE. Collectively, these findings demonstrate that treatment with UA-8 significantly enhances viability of HL-1 cells during starvation allowing cells to recover from injury. Further evidence of protection was observed following 24 h starvation where HL-1 cells treated with UA-8 were still beating indicating retention of functional activity (Figure 2.2B).

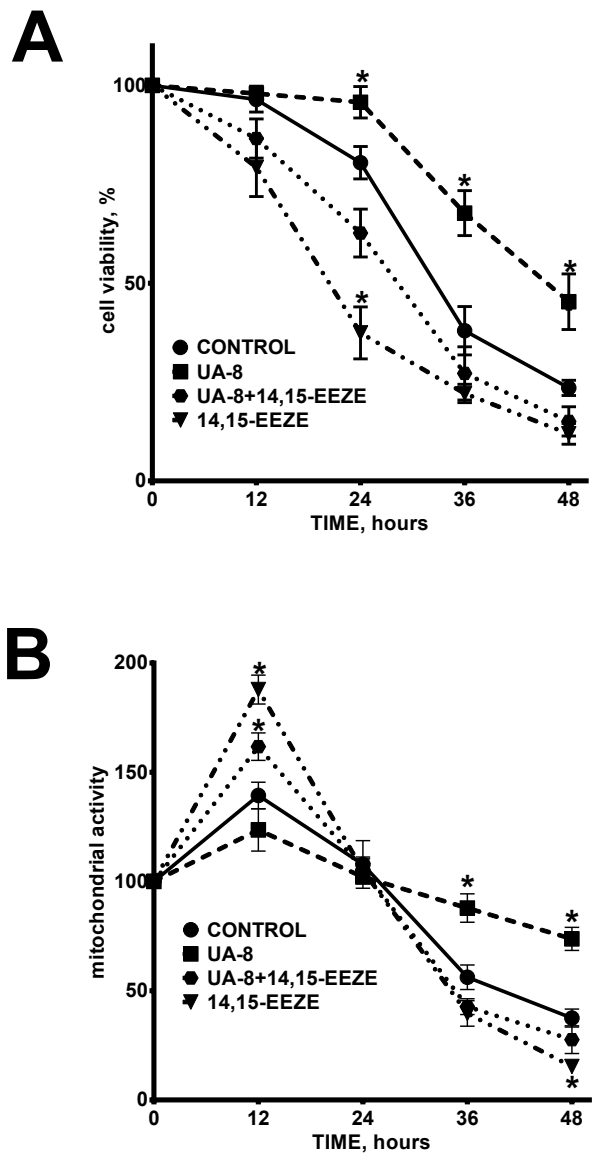


Figure 2.1 Survival of HL-1 cells during 48 h of starvation

HL-1 cells were treated with UA-8 (1 μM) in the presence or absence of 14,15-EEZE (10 μM) in amino acid-free and serum-free starvation buffer. (a) Cell viability was assessed by Trypan blue exclusion. (b) Total mitochondrial activity was measured by MTT assay. Values are represented as mean±S.E.M., N=3. Significance was set at $P<0.05$, *significantly different from control nonstarvation.

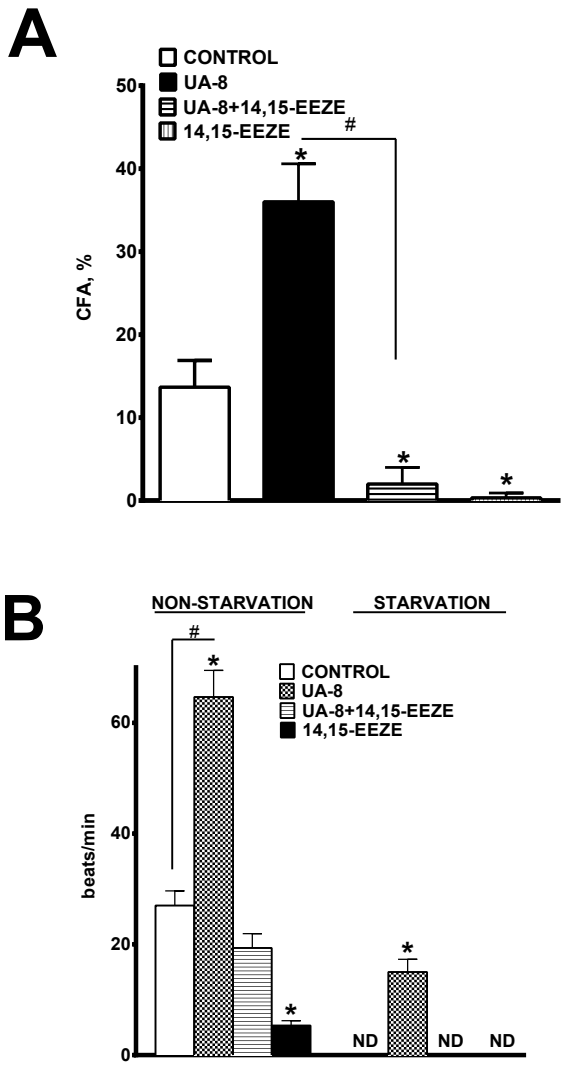


Figure 2.2 Functional recovery of HL-1 cells in response to UA-8 treatment

(a) Alterations in colony formation ability of HL-1 cells starved for 24 h with and without UA-8. (b) Effect of UA-8 on contractility of HL-1 cells starved for 24 h. Values are represented as mean±S.E.M., N=3. Significance was set at $P<0.05$, *significantly different from control nonstarvation or statistically not different (ND), $^{\#}$significantly different from UA-8.

2.3.2 UA-8 ameliorates detrimental effects of starvation

Starvation initiates a very complex, yet poorly understood stress response. Therefore, we sought to unravel the possible mechanisms involved in cell death during starvation and whether UA-8 could affect the cell death process. Accordingly, we measured alterations in caspase-3 and proteasomal activities in HL-1 cells during starvation to assess overall cellular injury. Starvation is known to trigger release of apoptogenic factors inducing cell death. Thus, we determined the apoptotic response in starvation-induced cell death. We observed that starvation induced a rapid activation of caspase-3, indicating the apoptotic response, which was significantly attenuated when cells were treated with UA-8 (Figure 2.3A).

Following extended starvation, cells begin to catabolize various complex molecules such as polysaccharides, nucleic acids and proteins to provide substrates for energy production. The accumulation of ubiquinated proteins followed by activation of 20S proteasome activity represents a marker of this cellular degenerative process (120). We, therefore, assessed 20S proteasome activity in starved HL-1 cells. Starvation induced a rapid increase in the level of 20S proteasome activity in HL-1 cells, which was significantly attenuated when cells were treated with UA-8 (Figure 2.3B). All together, the data demonstrate that UA-8 has a strong anti-degenerative effect toward starved cells. All protective effects of UA-8 were greatly diminished by co-treatment with 14,15-EEZE suggesting an intrinsic EET-mediated mechanism.

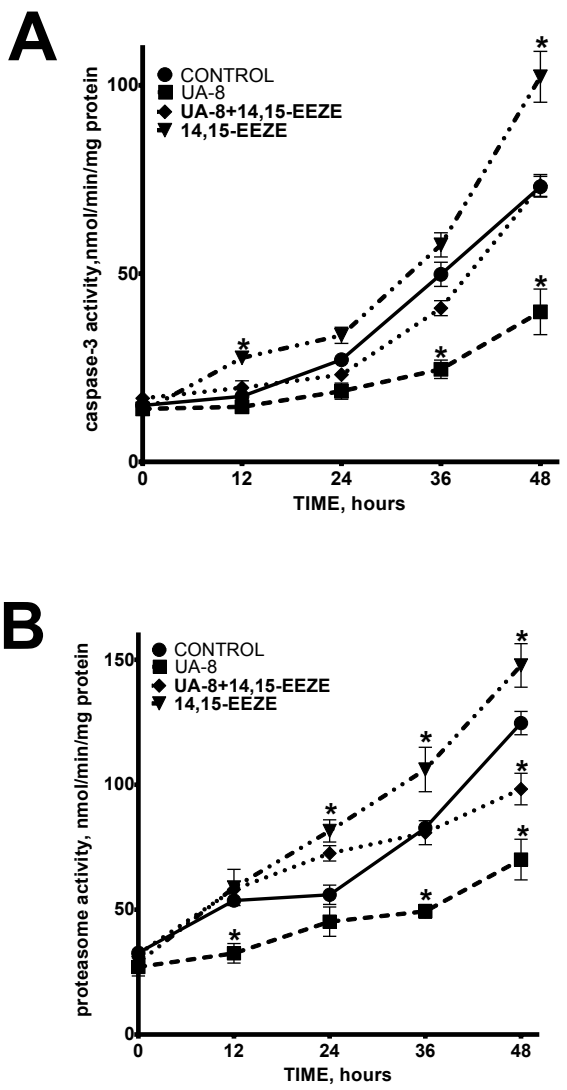


Figure 2.3 Effect of UA-8 treatment on starvation-induced cellular stress responses in HL-1 cells

(a) Changes in caspase-3 activity of HL-1 cells starved with and without UA-8. (b) Changes in total proteasome activity of HL-1 cells starved with and without UA-8. Values are represented as mean±S.E.M., N=3. Significance was set at $P<0.05$, *significantly different from control nonstarvation or statistically not different (ND).

2.3.3 Treatment with UA-8 prevented starvation-induced cellular stress responses in NCM

We subjected NCM to 24 h starvation following the same protocol used for HL-1 cells. Similarly, starvation triggered activation of both caspase-3 (Figure 2.4A) and proteasome activities in NCM (Figure 2.4B), and significantly reduced beating rate. Consistent with the data observed in HL-1 cells, treating NCM with UA-8 significantly reduced the adverse responses triggered by starvation. Again, co-treatment with 14,15-EEZE abolished the protective effects of UA-8.

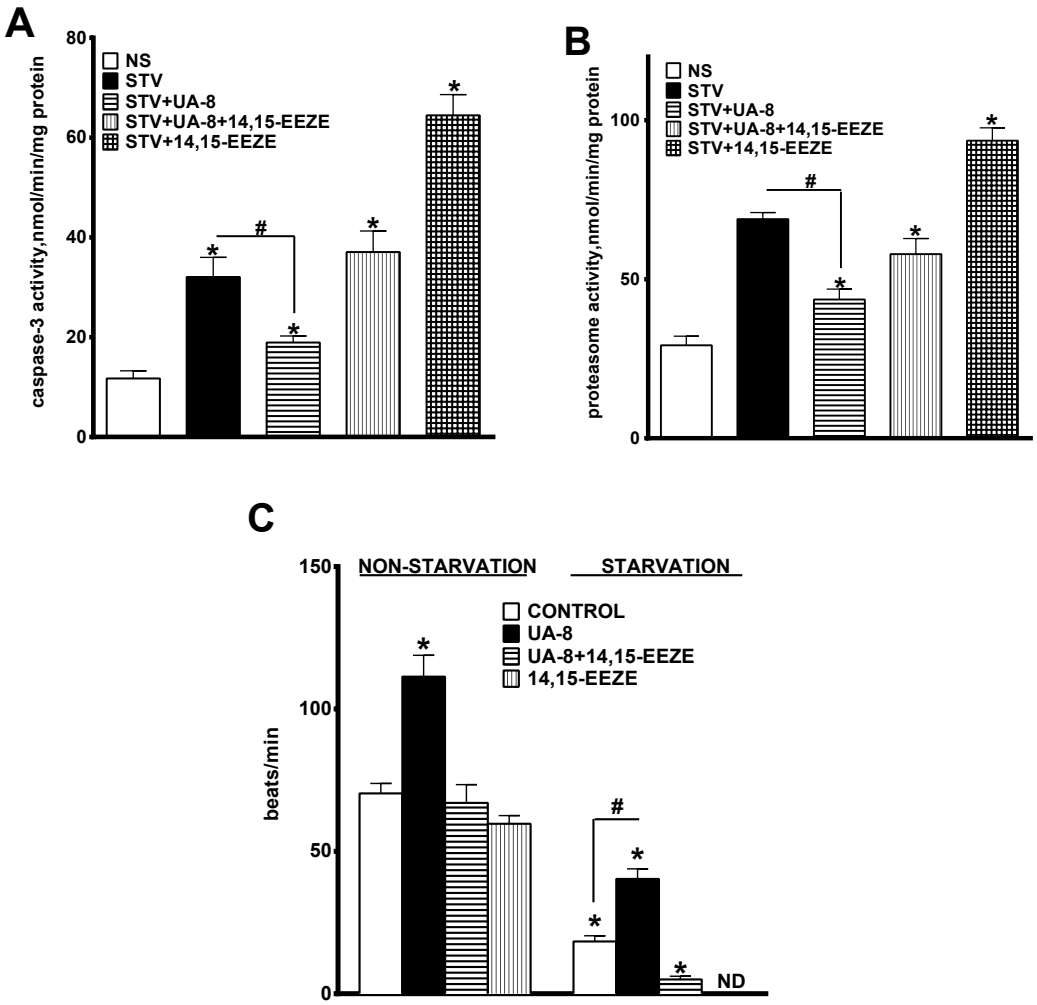


Figure 2.4 Effect of UA-8 treatment on starvation-induced cellular stress responses in NCM

NCM were treated with UA-8 (1 μM) in the presence or absence of 14, 15-EEZE (10 μM) in amino acid-free and serum-free starvation buffer for 24 h. Starvation induced activation of (a) caspase-3 and (b) proteasome activity in NCM. (c) Effect of UA-8 on contractility of NCM starved for 24 h. Values are represented as mean±S.E.M., N=3. Significance was set at $P<0.05$,

[*]significantly different from control nonstarvation or statistically not different (ND), [#]significantly different from UA-8.

2.3.4 UA-8 modulates the autophagic response in starved HL-1 cells

Cell survival during starvation has been shown to rely on activation of autophagy, which represents a major pathway regulating recycling of damaged molecules and organelles (108). Accordingly, it was reasonable to suggest that regulation of autophagy might represent an integral component of the UA-8 protective effect toward HL-1 cells during starvation. To our knowledge, no data have been published regarding the effect of eicosanoids on regulation of autophagy. Therefore, we assessed the level of autophagy in starved HL-1 cells. The formation of LC3-II protein and assembling of autophagosomes are important steps in the autophagic pathway. Figure 2.5 demonstrates that starvation rapidly up-regulated the levels of LC3-II in HL-1 cells during the first two hours of starvation followed by a slow decline until the end of starvation. Remarkably, treatment with UA-8 resulted in a constantly higher level of LC3-II expression in starved cells. Figure 2.5 shows results of western blot quantification after 2 and 24 h of starvation demonstrating a 5-fold increase in LC3-II expression in HL-1 cells treated with UA-8 during starvation. Furthermore, co-treatment with 14,15-EEZE significantly prevented UA-8 mediated effects on the autophagic response.

LC3-II has a crucial role in formation of autophagosomes, which are subsequently targeted to lysosomes. An individual autophagosome is represented as a punctum by immunofluorescence microscopy.

Autophagy is a dynamic process that involves a continual flux in healthy cells. Chloroquine is known to prevent the degradation of autophagosomes resulting in their accumulation within the cell. Chloroquine was used as a control treatment to demonstrate morphological hallmarks of autophagosomes. Treatment of HL-1 cells with chloroquine significantly increased the number of autophagosomes, while control cells had only a few puncta and very disperse intracellular fluorescence. Starvation triggered accumulation of autophagosomes in HL-1 cells (Figure 2.6A). Importantly, we observed that the formation of autophagosomes was robust and appeared merged in the cells treated with UA-8. There was a noticeable reduction in intracellular fluorescence compared to starvation control. Co-treatment with 14,15-EEZE attenuated the formation of autophagosomes in starved HL-1 cells treated with UA-8. Together, these data suggest that UA-8 treatment results in formation of LC3-II and accumulation of autophagosomes. Mechanistically, it is possible that UA-8 might block autophagic flux in starved cells. However, given the fact that autophagy represents a mechanism of cell survival we hypothesize that the protective effect of UA-8 rather involves activation of autophagy. Also, electron micrograph images revealed autophagosomal bodies in HL-1 cells following 24 h of starvation and UA-8 treatment, with some vacuoles containing mitochondria (Figure 2.6B). Mitochondria were dense and contained compact cristae correlating with increased function.

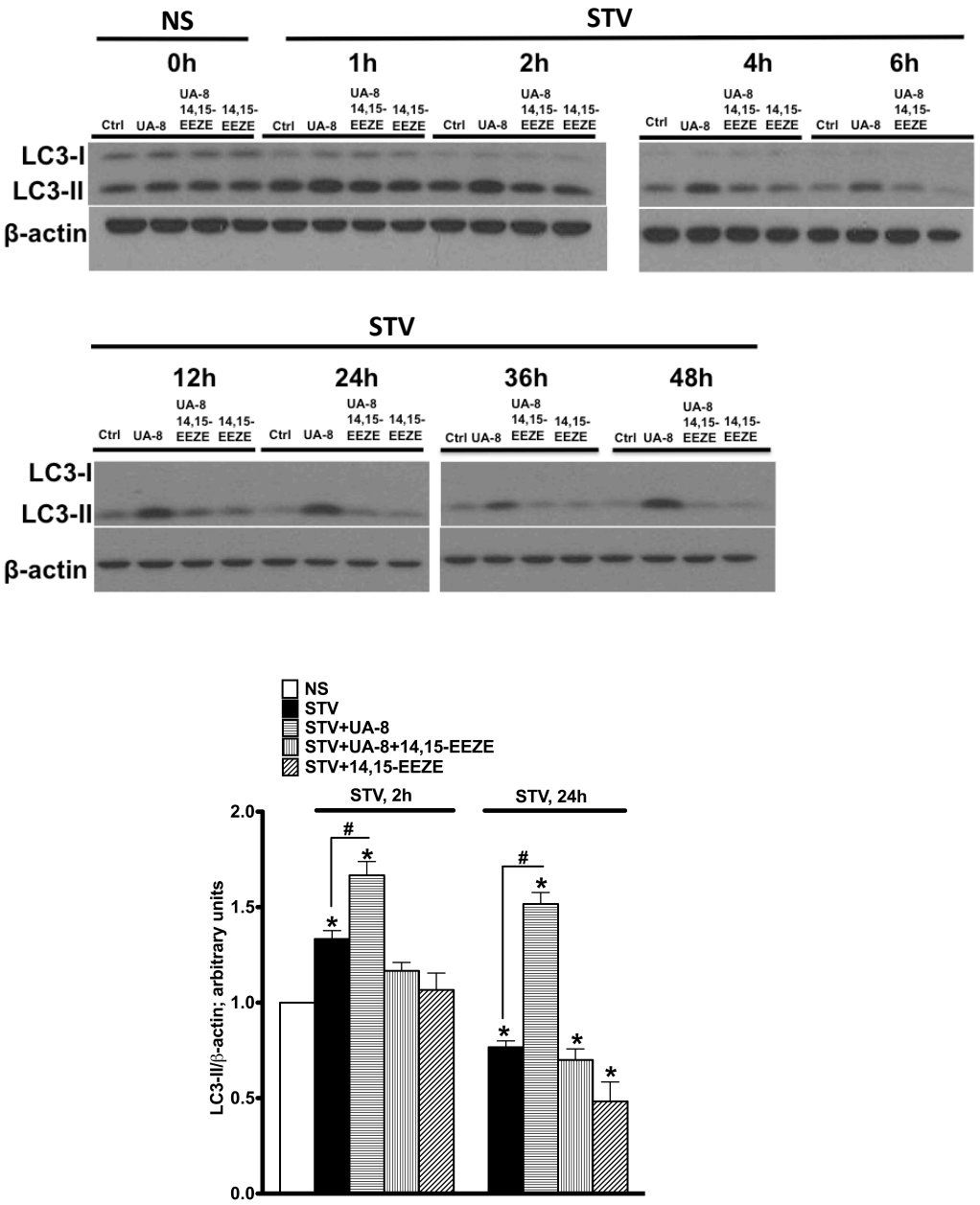
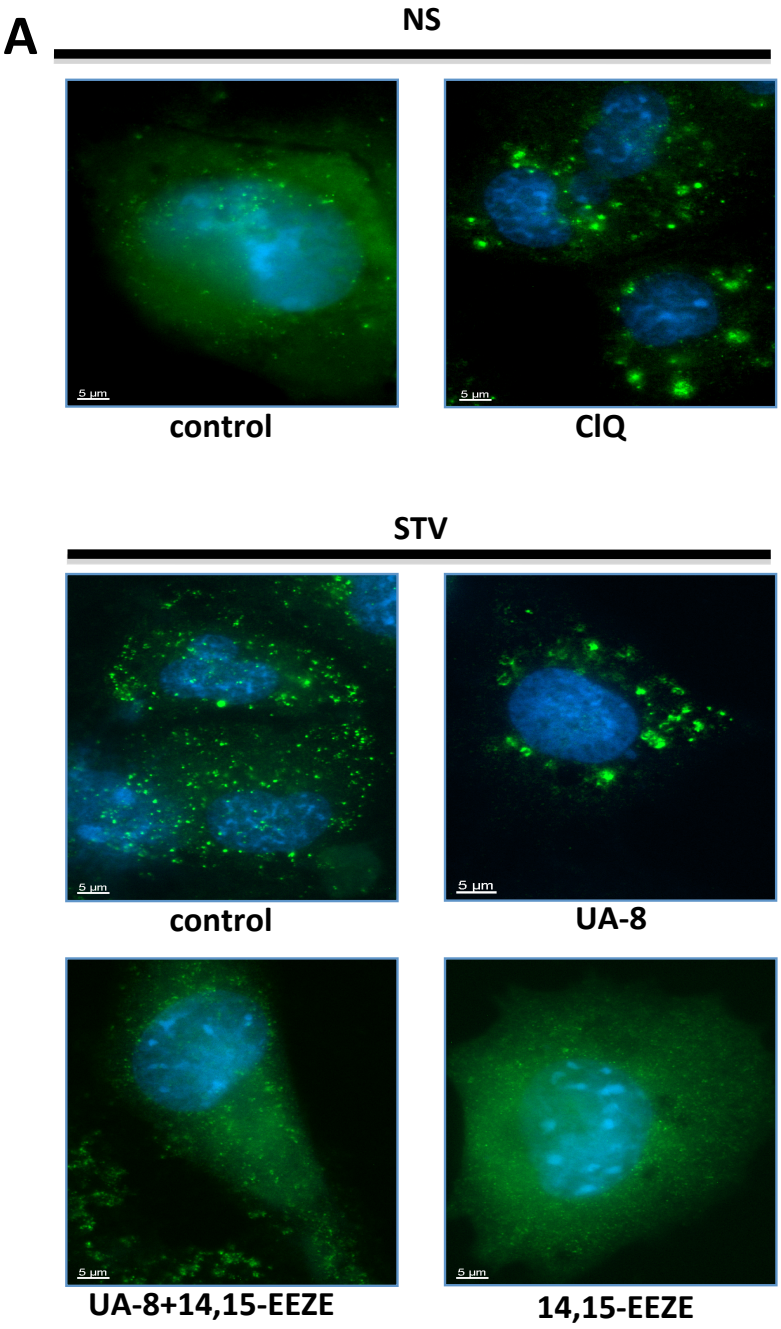


Figure 2.5 Enhancement of the autophagic response with UA-8 treatment in HL-1 cells during starvation

Formation of LC3-II protein in starved HL-1 cells. Left panel: representative western blots demonstrating the time course accumulation of LC3-II in starved cells. Right panel shows the results of western blot

quantification after 2 and 24 h of starvation, respectively. Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation, $^{\#}$significantly different from UA-8


A
NS
5 µm
control
5 µm
ClQ
STV
5 µm
control
5 µm
UA-8
5 µm
UA-8+14,15-EEZE
5 µm
14,15-EEZE

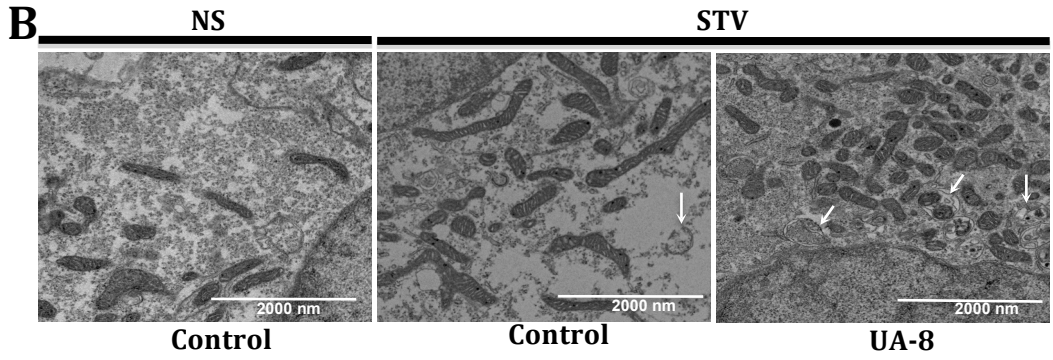


Figure 2.6 Enhancement of the autophagic response with UA-8 treatment in HL-1 cells during starvation (IHC and EM)

(a) Representative images following 24 h of starvation in HL-1 cells immunostained to detect LC3 positive puncta (green), a marker of autophagy. Nonstarved HL-1 cells were treated with chloroquine (50 μM), a blocker of autophagosomal degradation, as a control. Images were acquired with a Zeiss Axio Observer epifluorescence microscope using a X 63 objective (Oberkochen, Germany). Alexa Fluor 488 was conjugated LC3 Ab (green) and DAPI nuclear stain (blue) were utilized. (b) Representative electron micrograph (EM) images of nonstarved HL-1 cells and cells starved for 24 h with and without UA-8. White arrows identify autophagosomal vacuoles; note mitochondrial engulfment.

2.3.5. 14,15-EET limits starvation-induced Injury

To assess if the UA-8-mediated protective effects resemble those of EETs, we assessed the effect of 14,15-EET with and without 14,15-EEZE following 24 h starvation in HL-1 cells and in NCM (20). Similar to UA-8, 14,15-EET increased the levels of LC3-II in both HL-1 cells (Figure 2.7A) and NCM (Figure 2.7B) after 24 h of starvation suggesting there was activation of the autophagic response. Furthermore, treatment with 14,15-EET attenuated starvation-increased caspase-3 and proteasome activities in HL-1 cells (Figure 2.7C, D). Importantly, addition of 14,15-EEZE abolished all protective effects of 14,15-EET as observed with UA-8.

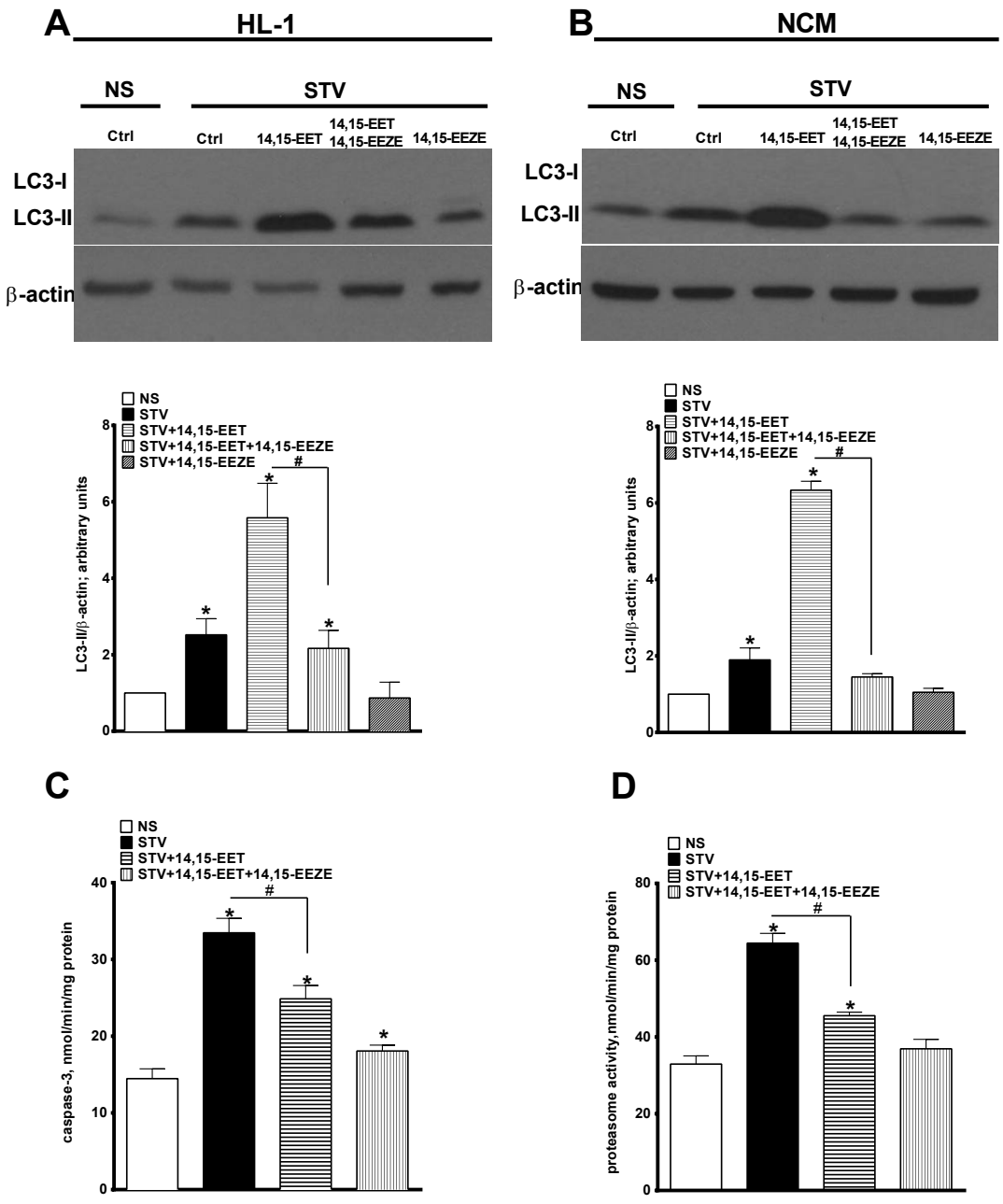


Figure 2.7 Recapitulation of the protective effects of UA-8 with 14,15-EET toward starved HL-1 cells and NCM

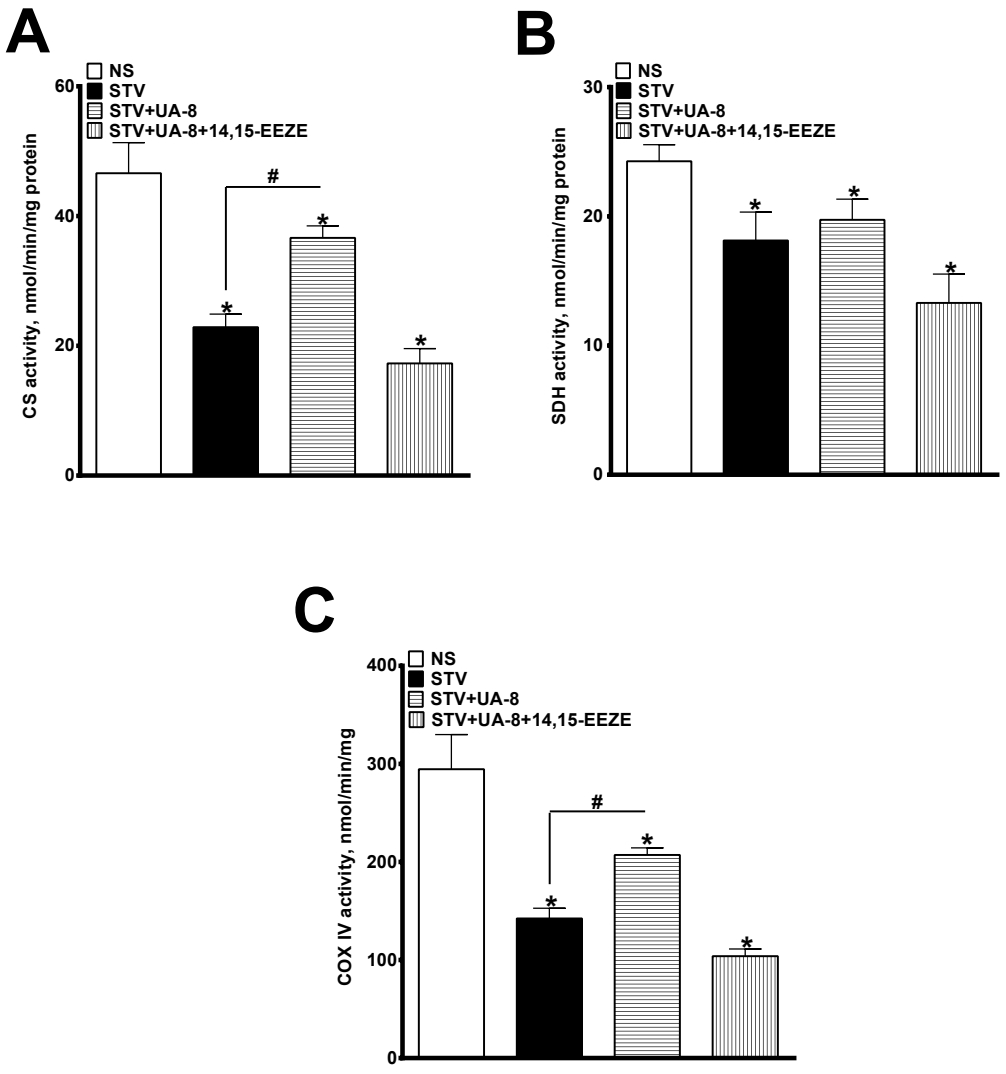
HL-1 cells and NCMs were starved for 24 h with or without 14,15-EET (1 μM). (a) Treatment with 14,15-EET increased the levels of LC3-II in starved HL-1 cells and (b) in NCMs as demonstrated in immunoblots and

quantified in corresponding histograms. (c) Treatment with 14,15-EET attenuated starvation-induced caspase-3 and (d) proteasome activities in starved HL-1 cells. Co-treatment with 14,14-EEZE (10 μM) abolished all observed protective effects of 14,15-EET. Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation, $^{\#}$significantly different from 14,15-EET.

2.3.6 UA-8 protects mitochondria function

In order to sustain cell viability and recover from injury, cellular responses to stress include steps that attempt to preserve mitochondrial integrity (57). To determine the impact of starvation on mitochondrial function, we assessed the activities of key enzymes reflecting the state of mitochondrial metabolic activity (167). We found that UA-8 prevented the decrease in CS, SDH and COX IV enzymatic activities observed in control groups following 24 h starvation, no significant protective effect was observed for SDH in HL-1 cells (Figure 2.8A-F). Next, we assessed the alterations in the expression of essential mitochondrial proteins during starvation. We found that NCM starved for 24 h had an elevated level of mitochondrial marker-proteins such as VIDAC, SDH and COX IV (Figure 2.9). This observation suggests that starved cardiac cells did not lose mitochondrial content. This observation is also reinforced by EM images (Figure 2.6B) where preservation of mitochondrial content during starvation is clearly demonstrated.

HL-1


A
NS
STV
STV+UA-8
STV+UA-8+14,15-EEZE
CS activity, nmol/min/mg protein
60
40
20
0
#
*
B
SDH activity, nmol/min/mg protein
30
20
10
0
C
COX IV activity, nmol/min/mg
400
300
200
100
0

NCM

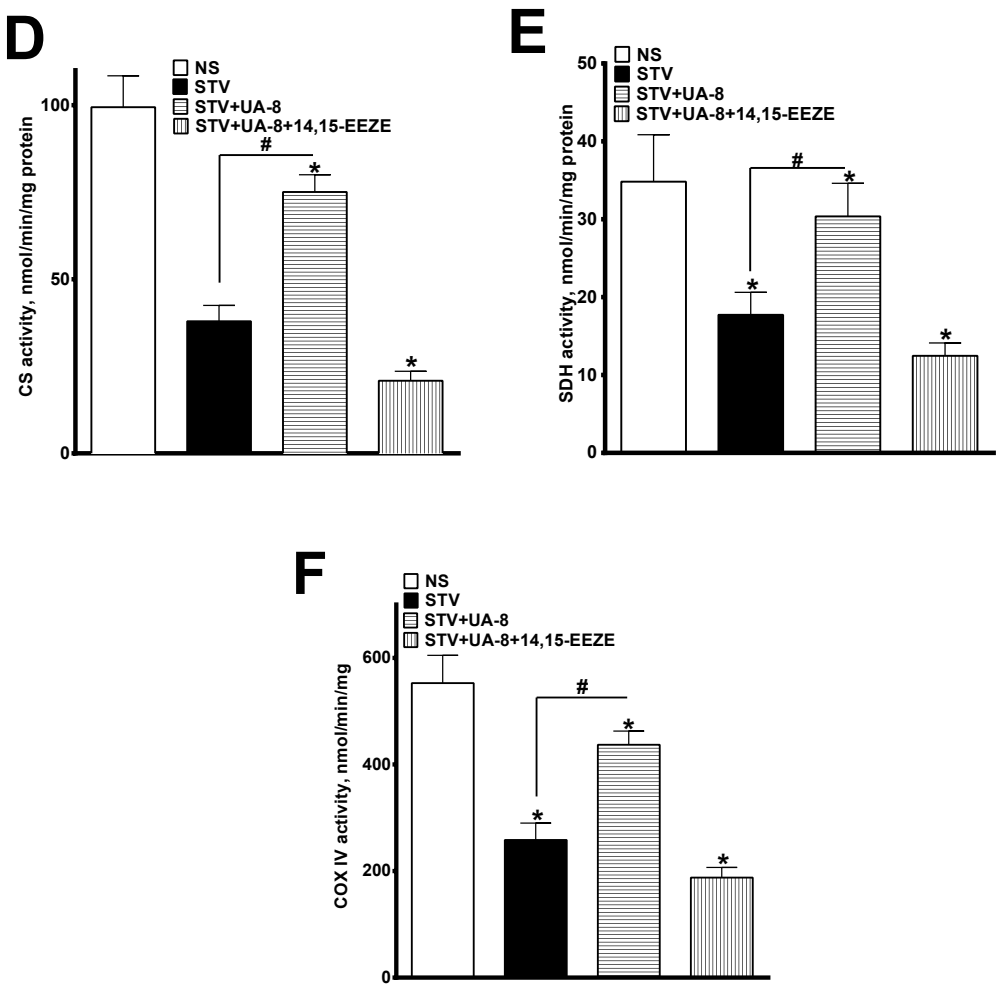

D
NS
STV
STV+UA-8
STV+UA-8+14,15-EEZE
CS activity, nmol/min/mg protein
100
50
0
#
*
E
SDH activity, nmol/min/mg protein
50
40
30
20
10
0
F
COX IV activity, nmol/min/mg
600
400
200
0

Figure 2.8 Treatment with UA-8 and maintaining of a healthy pool of mitochondria during starvation

Activities of key mitochondrial enzymes were assessed in HL-1 cells and NCM following 24 h of starvation. Citrate synthase (a, d), succinate dehydrogenase (b, e) and COX IV (c, f) activities were measured in HL-1 cells and NCMs in nonstarved (NS) and starved cells (24 h STV) treated with UA-8 (1 μM) or without 14,15-EEZE (10 μM). Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation, $^{\#}$significantly different from UA-8.

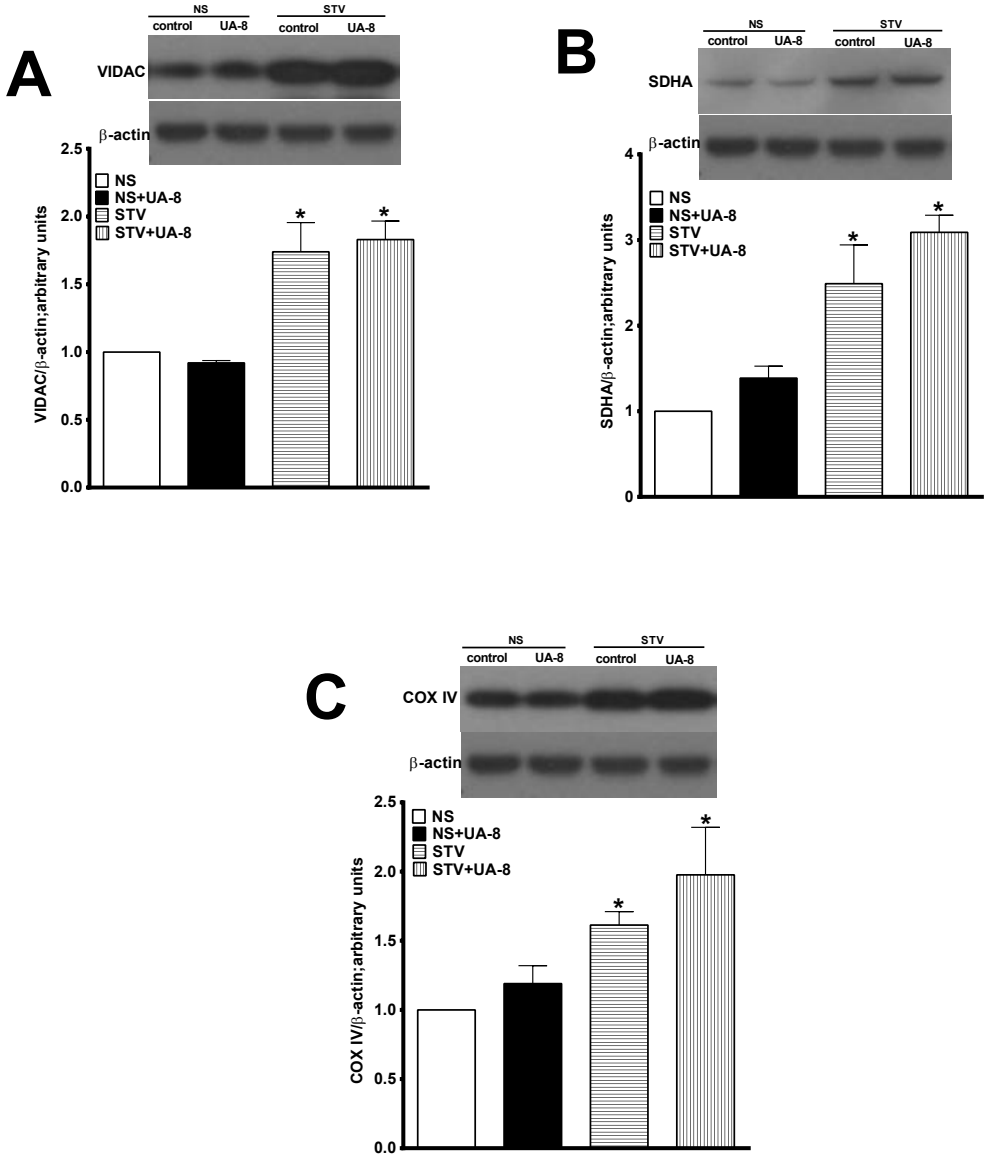


Figure 2.9 Mitochondrial proteins during starvation

Increased expression of mitochondrial proteins (a) VDAC, (b) succinate dehydrogenase and (c) COX IV in NCMs following 24 h of starvation were observed in both control and UA-8-treated cells, as detected by western blot. Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation.

2.3.7 UA-8 Protective effect modulates the autophagic response

In order to more precisely clarify the involvement of autophagy in the UA-8-mediated protective effect, we infected HL-1 cells with shRNA targeted to *Atg7* or nonspecific shRNA (SHAM). Atg7 is essential for autophagosomal formation (116). Silencing *Atg7* resulted in a significant decline in cell viability during starvation, where more than 80% of cells were dead at 24 h (Figure 2.10), which were no longer be protected by UA-8. Similar results were observed when caspase-3 (Figure 6C) and proteasome activities were assessed (Figure 2.11). Silencing of *Atg7* resulted in robust activation of both caspase-3 and proteasome activities in HL-1 cells after 12 h of starvation, which UA-8 failed to inhibit. In addition, *Atg7*-silencing significantly decreased LC3-II protein levels (Figure 2.12) suggesting autophagy was inhibited.

In order to further reinforce the outcome of *Atg7*-silencing experiments, we inhibited autophagy in HL-1 cells by using the pharmacological agent, 3-methyladenine (3-MA), which prevents formation of autophagosomes in mammalian cells (116). Figure 2.13 demonstrates that treatment with 3-MA (5mM) within 24 h abolished the UA-8-mediated inhibition of caspase-3 and total proteasome activities in starved HL-1 cells. Consistent with the above observations our data suggests that modulation of autophagy is an important component of UA-8 protective effects during starvation.

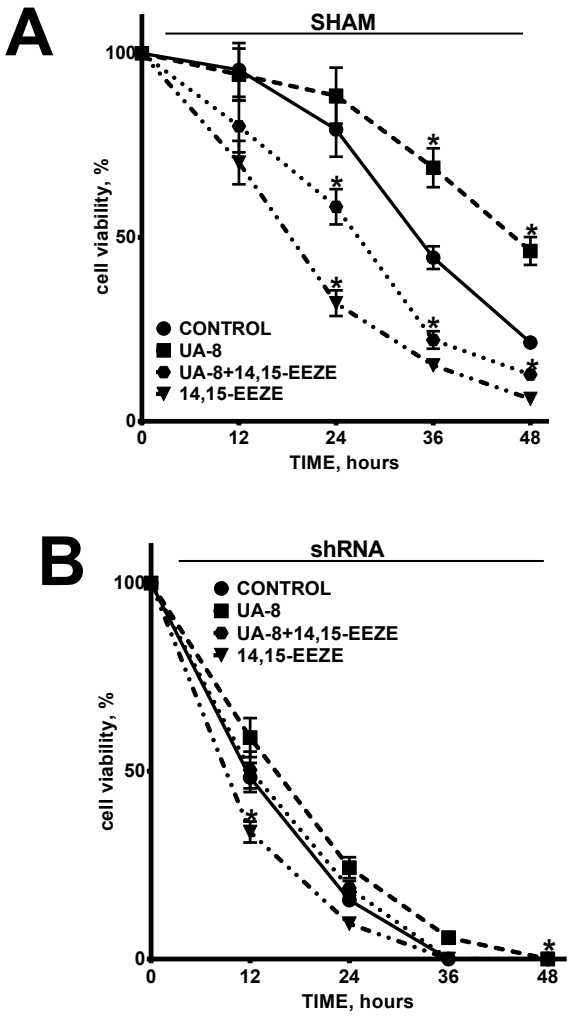


Figure 2.10 Genetic inhibition of the autophagic response and the UA-8-mediated improvement in cell survival

HL-1 cells were transfected with either shRNA to ATG7 or scrambled shRNA (Sham). (a, b) UA-8 (1 μM) failed to prevent the loss in cell viability in ATG7-silenced HL-1 cells as compared to sham treated cells. Values are represented as mean ±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control.

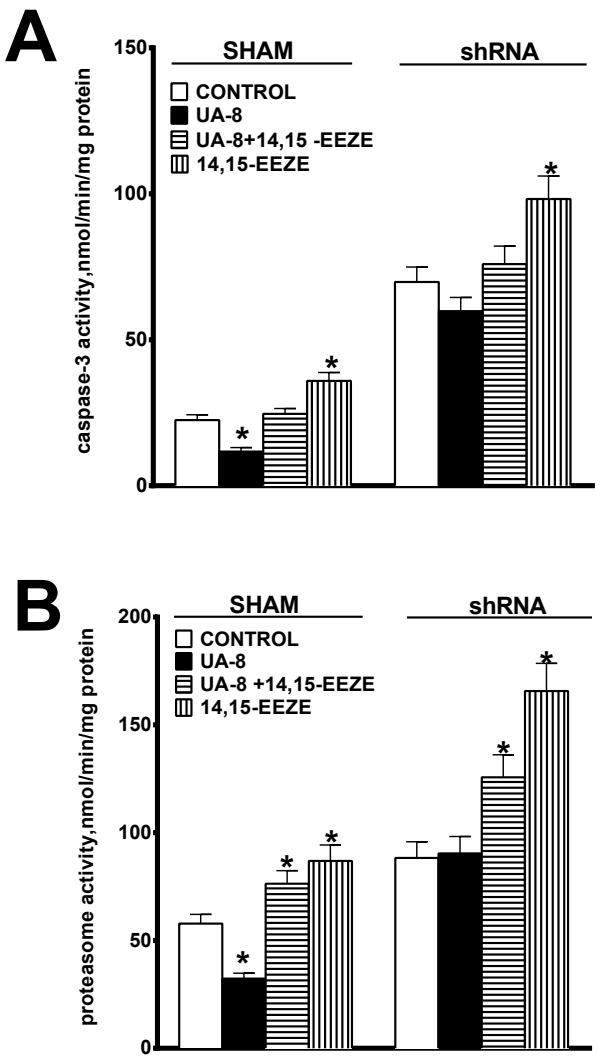


Figure 2.11 Genetic inhibition of the autophagic response and the UA-8-mediated reduction in caspase-3 and proteasome activities

(a) Silencing of ATG7 prevented UA-8 from limiting increases in caspase-3 and (b) total proteasome activities in starved HL-1 cells. Values are represented as mean ±S.E.M., N=3. Significance was $P<0.05$, * significantly different from control.

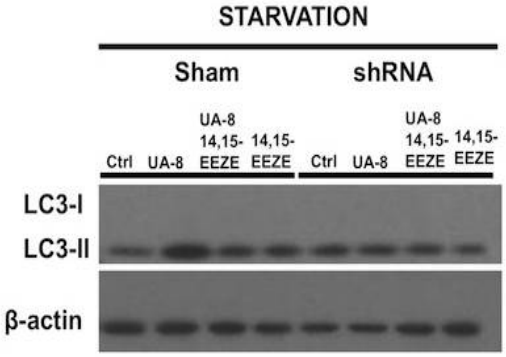


Figure 2.12 Silencing Atg7 and UA-8-mediated modulation of autophagy

A representative western blot of LC3-I and LC3-II expression after 24 h of starvation in sham and ATG7-silenced HL-1 cells showing 50–60% reduction in UA-8 enhanced autophagy.

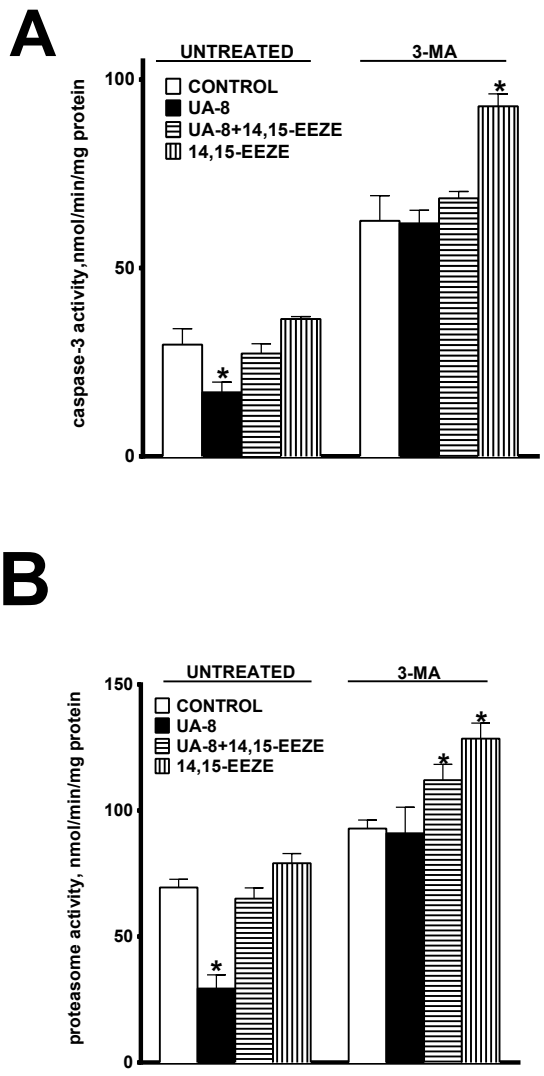


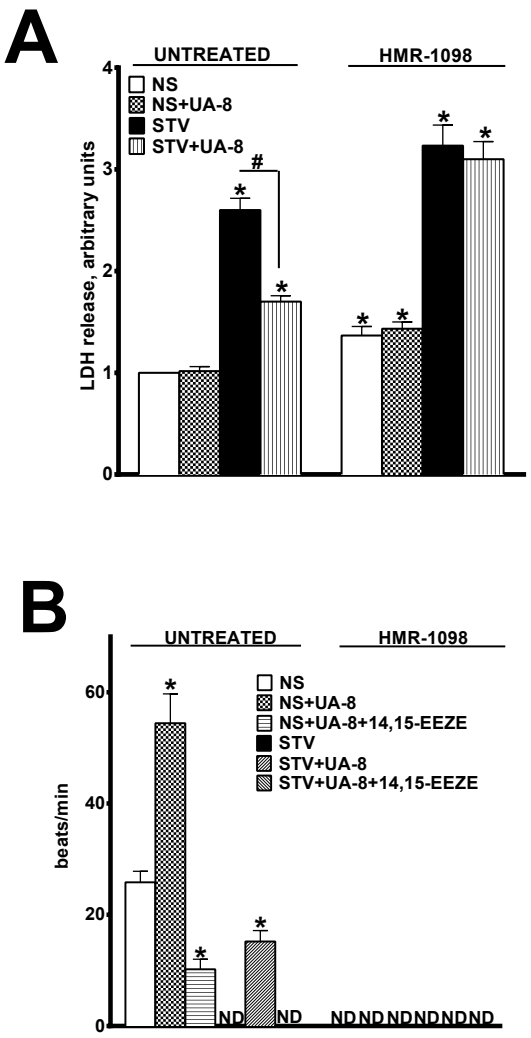
Figure 2.13 Pharmacological inhibition of the autophagic response and the UA-8-mediated effects

(a, b) HL-1 cells were starved in the presence of 3-MA (5mM), a pharmacological inhibitor of autophagy, for 24 h. 3-MA reduced the protective effects of UA-8 toward caspase-3 and total proteasome activities in starved HL-1 cells. Values are represented as mean ±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control.

2.3.8 UA-8 protective effect is mediated by ATP-Sensitive K^+ channels

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 (67, 168). EETs have been shown to be activators of *pm*K_{ATP} channels impacting mitochondrial function (7, 103). To determine if UA-8 mediated effects occur via *pm*K_{ATP} channels, both HL-1 cells and NCM were treated with HMR-1098 (10μM), a *pm*K_{ATP} channel selective inhibitor, under starvation conditions for 24 h (Figure 2.14). Inhibition of *pm*K_{ATP} channels with HMR-1098 prevented UA-8 mediated cellular protection against starvation induced injury in HL-1 cells resulting in increased LDH release, proteasome and caspase-3 activities while decreasing the beating rate (Figure 2.14). Consistent, with the response in HL-1 cells, we observed that inhibition of *pm*K_{ATP} channels resulted in a significant loss of UA-8 protective effects in NCM during starvation (Figure 2.15).

HL-1


A
UNTREATED
HMR-1098
NS
NS+UA-8
STV
STV+UA-8
LDH release, arbitrary units
0
1
2
3
4
*
#
B
UNTREATED
HMR-1098
NS
NS+UA-8
NS+UA-8+14,15-EEZE
STV
STV+UA-8
STV+UA-8+14,15-EEZE
beats/min
0
20
40
60
ND
ND
ND ND ND ND ND ND

HL-1

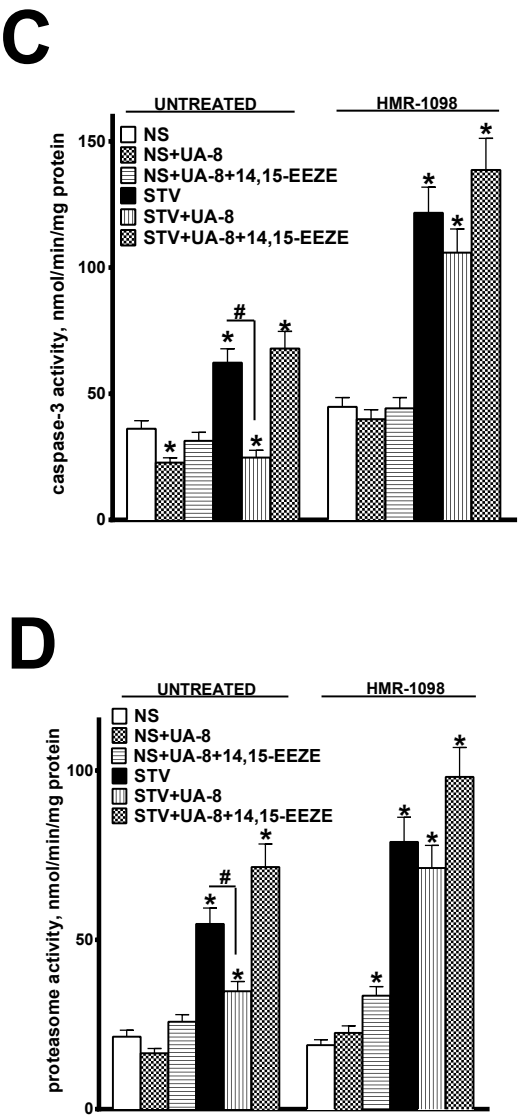
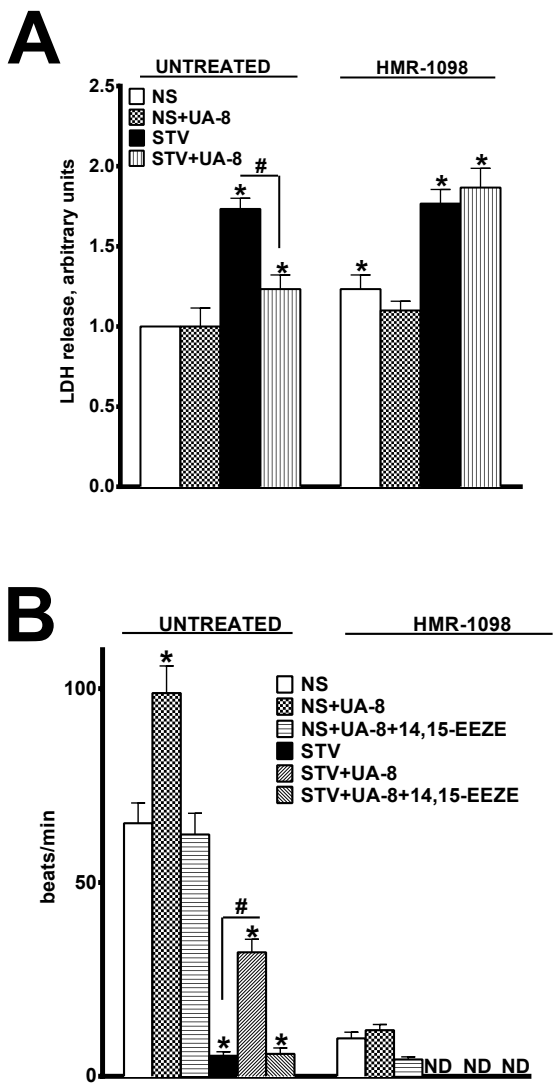


Figure 2.14 Inhibition of *pm*K_{ATP} channels and the UA-8-mediated protective effects in starved HL-1 cells

HL-1 cells were starved for 24 h in the presence of UA-8 (1 μM) with or without HMR-1098 (10 μM), a pharmacological inhibitor of *pm*K_{ATP} channels. (a) Treatment with UA-8 reduced release of LDH from starved HL-1 cells indicative of increased cell survivability. (b) HMR-1098 abolished stimulating effect of UA-8 on contractility in HL-1 cells under

normal conditions and after 24 h of starvation. (c, d) Inhibition of *pm*K_{ATP} channels with HMR-1098 significantly abolished the ability of UA-8 to prevent activation of caspase-3 and proteasome activity in starved HL-1 cells. Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation, $^{\#}$significantly different from UA-8 treatment or statistically not different (ND).

NCM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HMR-1098
NS
NS+UA-8
STV
STV+UA-8
LDH release, arbitrary units
2.5
2.0
1.5
1.0
0.5
0.0
#
*
B
UNTREATED
HMR-1098
NS
NS+UA-8
NS+UA-8+14,15-EEZE
STV
STV+UA-8
STV+UA-8+14,15-EEZE
beats/min
100
50
0
#
*
ND ND ND

NCM

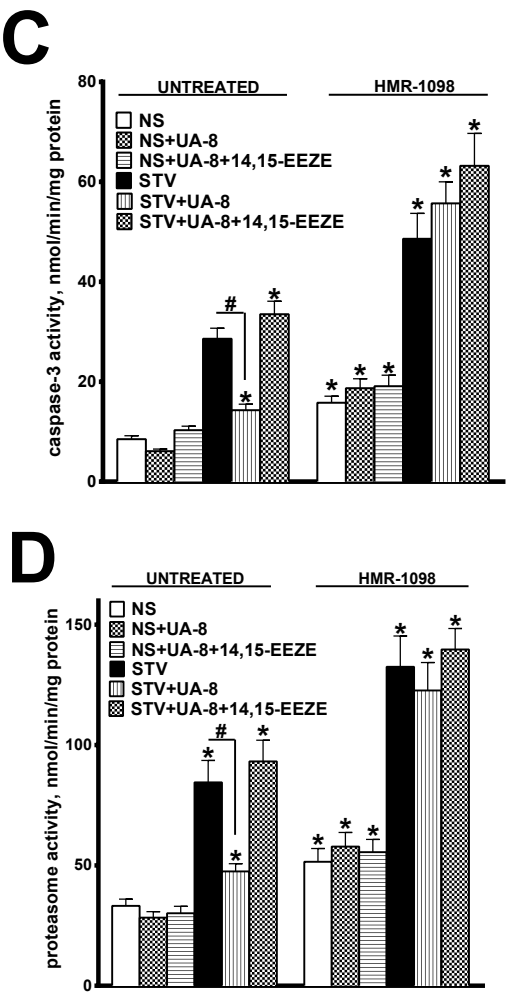


Figure 2.15 Inhibition of *pm*K_{ATP} channels UA-8-mediated protective effects in starved NCM

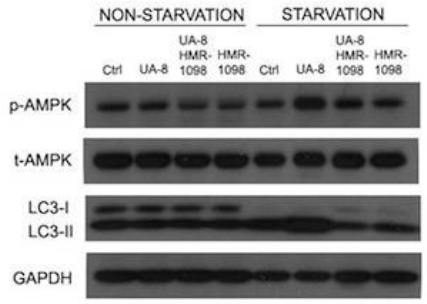
NCM were starved for 24 h in the presence of UA-8 (1 μM) with or without HMR-1098 (10 μM), a pharmacological inhibitor of *pm*K_{ATP} channels. (a) Treatment with UA-8 reduced release of LDH from starved NCM indicative of increased cell survivability. (b) HMR-1098 abolished stimulating effect of UA-8 on contractility in NCM cells under normal conditions and after 24 h of starvation. (c, d) Inhibition of *pm*K_{ATP} channels with HMR-1098

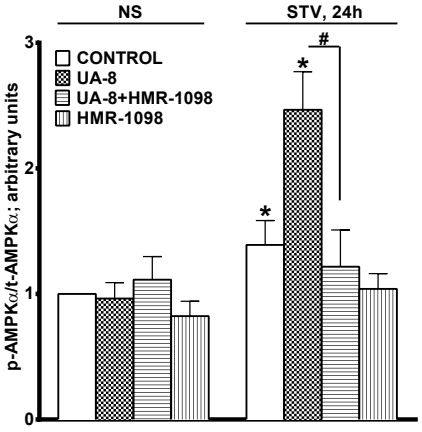
significantly abolished the ability of UA-8 to prevent activation of caspase-3 and proteasome activity in starved NCM. Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation, $^{\#}$significantly different from UA-8 treatment or statistically not different (ND).

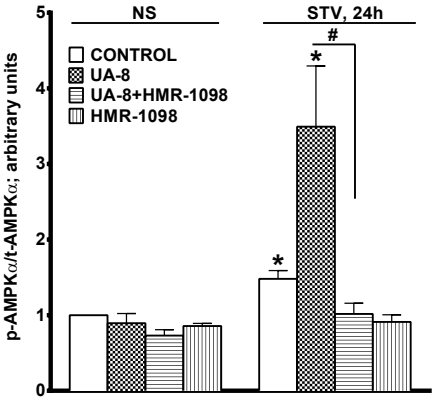
2.3.9 Activation of AMPK and modulation of the autophagic response in starved cells by UA-8 was abolished by co-treatment with HMR-1098

AMP-activated protein kinase (AMPK) is a key metabolic sensor strongly activated under conditions of nutrient deprivation, such as during ischemia, which has a role in regulating cell proliferation and cell death. In both HL-1 cells and NCM, treatment with UA-8 resulted in a significant increase in phosphorylated AMPK following 24 h of starvation. This correlated with a marked increase in LC3-II levels (Figure 2.16). Importantly, inhibition of *pm*K_{ATP} channels with HMR-1098 abolished the UA-8-mediated activation of AMPK and increase in the levels of LC3-II (Figure 2.16).

A


HL-1
NON-STARVATION
STARVATION
Ctrl
UA-8
UA-8 HMR-1098
HMR-1098
p-AMPK
t-AMPK
LC3-I
LC3-II
GAPDH


NS
STV, 24h
CONTROL
UA-8
UA-8+HMR-1098
HMR-1098
p-AMPKα/t-AMPKα; arbitrary units
0
1
2
3
*
*
#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STV, 24h
CONTROL
UA-8
UA-8+HMR-1098
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p-AMPKα/t-AMPKα; arbitrary units
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4
5
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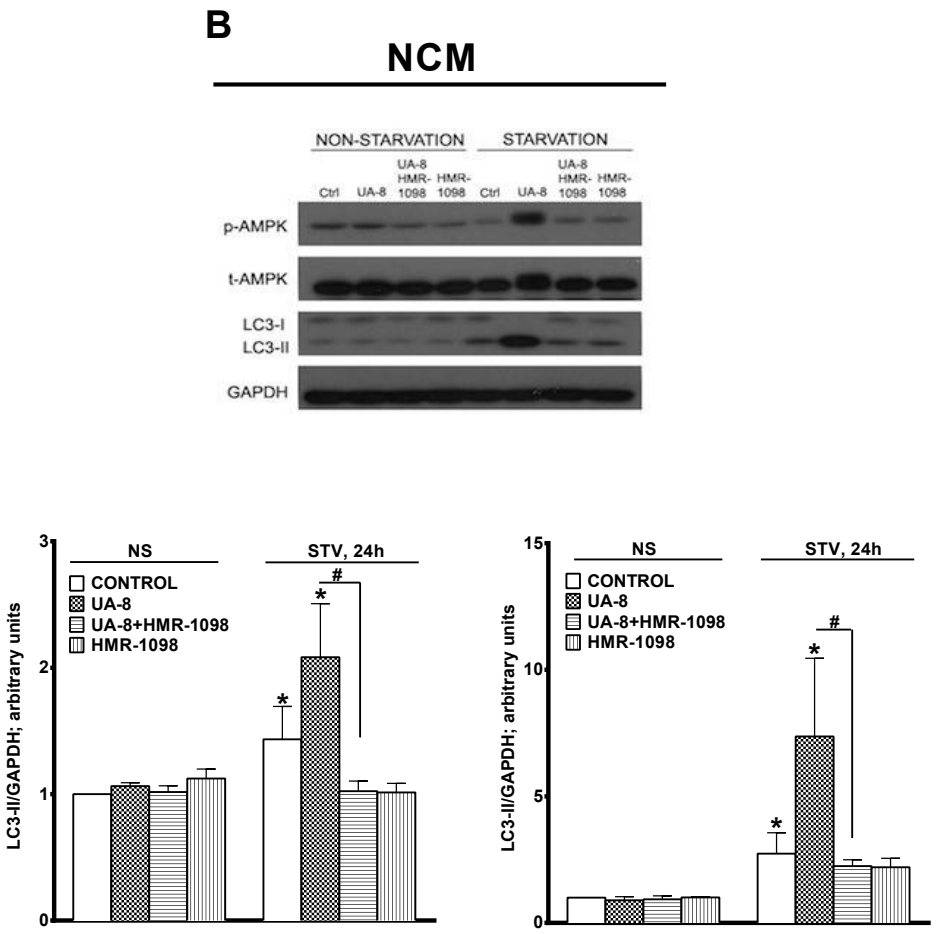


Figure 2.16 Inhibition of *pm*K_{ATP} channels, activation of AMPK and UA-8-mediated modulation of the autophagic response in starved HL-1 cells and NCM

(a) The increased phosphorylated AMPK (Thr172) correlated with UA-8-activated autophagic response following 24 h of starvation in HL-1 cells and (b) NCMs, which was detected by western blot. The relative changes in phosphorylated AMPK and LC3-II expression levels were quantified in HL-1 cells and NCMs following treatments after 24 h of starvation and are presented below as respective representative western blots.

Values are represented as mean±S.E.M., N=3. Significance was $P<0.05$, *significantly different from control nonstarvation, $^{\#}$significantly different from UA-8. t-AMPK: total AMPK.

2.4. DISCUSSION

In this study, we showed that EET-mediated events protect cardiac cells during starvation. The protective effect reduced caspase-3 AND proteasomal activities, which significantly improved cell viability and recovery of starved cardiac cells. Interestingly, the EET-mediated protective effect modulated the autophagic response, thus shifting the cell death process to promote cell survival. Mechanistic data suggested the signaling pathway involved *pm*K_{ATP} channels and activation of AMPK in starved HL-1 cells and NCM.

Starvation represents a unique biological situation, where activation of autophagy and apoptosis occur simultaneously (108). Therefore, predomination of autophagy (cell survival) over apoptosis (cell death) will result in a greater rate of cell survival or, in contrast, strong activation of an apoptotic signal will increase cell death (42). In our experimental model, we observed UA-8 significantly improved viability of both HL-1 cells and NCM following starvation. The protective effect was abolished by co-treatment with its antagonist 14,15-EEZE, suggesting the effects were EET specific, consistent with our previously published data (8). One of our key experiments demonstrated that UA-8 promoted greater colony formation of starved HL-1 cells compared to controls. Importantly, the CFA experiments started with the same number of cells and devoid of UA-8 suggesting the EET-mediated protective effect occurred during the starvation period. The pro-survival capability of UA-8 is consistent with

previous evidence demonstrating EET-mediated pro-carcinogenic effects (130).

Activation of degenerative processes has been described and attributed to detrimental consequences of prolonged starvation (78, 108, 137). Consistent with this evidence, starvation triggered a marked increase in caspase-3 and total proteasome activities in both HL-1 cells and NCM. We show that UA-8 significantly attenuated caspase-3 and total proteasome activation. Activation of autophagy has been shown to favor cell survival and suppress cell death under various stress conditions (10, 36, 54, 72, 119, 136, 147). While EETs are known to promote cell survival (9, 106), there is remarkably little known regarding their role in regulating autophagic pathways. We show that EET-mediated events increase expression of LC3-II and formation of autophagosomes (morphological data) in starved HL-1 cells. Furthermore, shRNA silencing of *Atg7*, an essential autophagic protein, abolished the protective effects of UA-8 and resulted in a significant decline in cardiac cell survival during starvation. The subsequent large increase in caspase-3 and proteasome activities, which occurred in cells where *Atg7* was silenced suggests there was a switch in cell death pathways occurred from autophagy to apoptosis. Taken together, our data strongly suggest that EET-mediated protective events involve modulating an autophagic response, which in turn promotes cell survival during starvation. While the exact mechanism remains unknown and might potentially involve blocking the autophagic

flux; we hypothesize that the protective effect involves activation of autophagy.

AMPK has a critical role in regulating cellular growth and metabolism, acting as a metabolic sensor allowing adaptive responses to reduced energy. Upstream factors such as LKB1 and CaMKKβ regulate AMPK activity under normal and stressed conditions, respectively (114). Activation of AMPK can trigger downstream signals directly activating ULK1 and inhibiting mTORC1 resulting in autophagy (69). Indeed, increased AMPK activation correlated with the enhanced levels of LC3-II protein and an increased number of autophagosomes in UA-8 treated cells, which was attenuated with HMR-1098. We, and others, previously demonstrated EET-mediated effects involve *pm*K_{ATP} channels; however, it is unknown how these channels regulate autophagy or AMPK activation (7, 35, 61, 102). Cardiac *pm*K_{ATP} channels are known to be involved in regulating ionic homeostasis under conditions of metabolic stress and have demonstrated cardioprotective effects (67, 168). *pm*K_{ATP} channels can be activated when cytoplasmic ATP is depleted, leading to shortening of action potential, reduced membrane depolarization, consequently reducing intracellular calcium overload (193). Currently, it remains unknown through which molecular mechanism(s) EETs target the autophagic response; our data suggest an important role for *pm*K_{ATP} channels in the EET-mediated modulation of an autophagic response.

Collectively, our data strongly suggest a regulatory role for EETs in autophagic signaling, which promotes cell survival. Interestingly, activation of AMPK has been shown to trigger removal of damaged mitochondria via ULK1-dependent mechanism and promotes biogenesis through a PCG-1α dependent process maintaining mitochondrial homeostasis following cellular stress (114). We previously demonstrated that EETs preserve mitochondria function and reduce damage to stress improving cell survival and limiting tissue injury (8, 9, 22, 39, 87). Mitochondria play a crucial role in cell survival during unfavorable conditions, including starvation; as such, their preservation is an important physiological strategy orchestrating cell survival and sustainability (57, 167). Our data demonstrated that mitochondrial content was preserved in starved cells following both control and UA-8 treatments. Importantly, the corresponding decline in mitochondrial function observed in controls was preserved by EET-mediated events. We speculate that the accumulation of mitochondrial protein content reflects the cells response to spare mitochondria from the degradation while the other cytosolic constitutes remain vulnerable to be degraded via the autophagic machinery. We can conclude that the mitochondria found in UA-8 treated cells were healthier. We hypothesize therefore, that EET-mediated events trigger a protective mechanism resulting in sustaining a healthier pool of mitochondria promoting cell survival. However, it remains unknown how EETs protect mitochondria in this model. While we did not observe direct activation of mitophagy, we

can infer that the EET-mediated protective mechanism(s) either promote the removal of damaged mitochondria or, alternatively, directly sustain mitochondrial function by enhancing the electron transport chain.

Thus, we hypothesize that EET-mediated events protect mitochondrial quality by regulating an autophagic response, preserving mitochondria and shifting the cell death pathway toward survival. Finely balanced autophagic machinery is important for proper function of terminally differentiated cardiomyocytes as loss of cardiomyocytes via apoptosis or necrosis would compromise cardiac function on the systemic level. In conclusion, we provide evidence that biological effects of eicosanoids are tightly interconnected with autophagy and the preservation of a pool of healthy mitochondria (Figure 2.17). This interconnection might be involved in pathogenesis of many diseases, therefore, can be considered as an attractive target for novel therapeutic interventions.

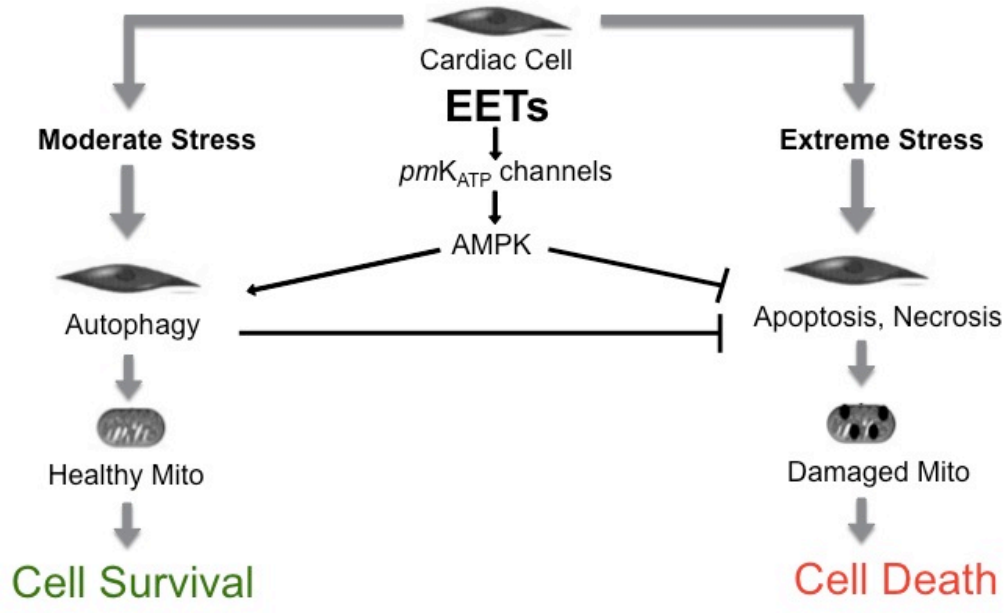


Figure 2.17 A general scheme illustrating a hypothesis for EET-mediated protective effects

Increased levels of EETs can shift cell death pathways from apoptotic and necrotic responses, which result in cell loss, to an autophagic pathway, resulting in cell survival. Autophagy may enhance turnover of damaged molecules and organelles, such as mitochondria, increasing survivability.

CHAPTER 3

CONCLUDING REMARKS AND FUTURE DIRECTIONS

3.1 CONCLUDING REMARKS

In this thesis, we sought to understand the mechanisms of EET-induced protection in cardiac cells. Despite evidence suggesting that EETs activate multiple signal transduction pathways (143, 165), the interrelation between these pathways and the exact molecular mechanism(s) remain poorly understood. Therefore, illustrating the underlying mechanisms of EET-mediated cardioprotection could reveal potential targets for novel therapeutic intervention.

The studies presented in this thesis demonstrate a novel molecular mechanism for EET-mediated cardioprotective effects. We provided evidence for the first time that EET-mediated protective effects involve modulation of an autophagic response. Furthermore, we found that EET-mediated modulation of the autophagic response was associated with a decrease in the apoptotic cell death, which is in agreement with previous reports (12, 35, 192). Most importantly, our findings demonstrated that EET-induced modulation of autophagy is necessary for mediating the cardioprotective effects of EETs.

Our group as well as others showed previously that the EETs cardioprotective effects involve activation of K_{ATP} channels (7, 61, 87, 102). Interestingly, and in agreement with these reports, we found, in the starvation model, that activation of *pm*K_{ATP} channels might be involved in the EETs cardioprotective effects including modulation of an autophagic response. Interestingly, we found that EET-mediated modulation of an

autophagic response is associated with phosphorylation of AMPK, an important regulator of the cell metabolic activity and autophagy (114).

Although our findings do not directly demonstrate how EETs modulate autophagy, we believe that EETs rather activate an autophagic response due to the following reasons: (1) treatment with EETs enhanced the cell viability throughout 48 hours of starvation whereas treatment with chloroquine, a known inhibitor of autophagy which blocks the fusion of autophagosomes with lysosomes (148), resulted in cell death within a few hours; and (2) treatment with EETs caused no change to the autophagic response in the non-starved cells, highlighting another difference to chloroquine-mediated inhibition of autophagy.

The involvement of mitochondria in the EET-mediated cardioprotection following myocardial IR injury has been previously described (7, 61, 87). Despite using a different model of cellular stress in our current studies, we found, in accordance with the previous reports (7, 9, 99, 204), that EETs preserved mitochondrial content and activity.

Collectively, we present in this thesis a mechanisms of EET cardioprotection is starved cardiac cells. For the best of our knowledge, we are the first to report that EET-mediated cardioprotective effects involve modulation of an autophagic response. Our mechanistic studies demonstrate that phosphorylation of AMPK is correlated with EET-mediated modulation of autophagy and that activation of *pm*K_{ATP} channels might be involved in the cardioprotective effects of EETs. Taken together, these data provide new insights into EET-mediated protection in starved cardiac cells.

3.2 FUTURE DIRECTIONS

EETs have long been recognized for mediating various biological events in the different body tissues and most notably in the cardiovascular and renal systems (82, 143). We are interested in understanding the underlying molecular mechanisms of EETs following myocardial IR injury. We, and others, showed previously multiple mechanisms for the EETs cardioprotective effects following myocardial IR injury (125). Particularly, our group showed that EETs improved post-ischemic functional recovery, minimized mitochondrial damage and delayed the loss of mitochondrial membrane potential (9, 87, 204). In this thesis, we demonstrated that EETs modulated an autophagic response in starved cardiac cells. We found that an increase in the autophagic response was correlated with increased phosphorylation of AMPK. Interestingly, and consistent with our previous studies (7), we demonstrated that activation of *pm*K_{ATP} channels is required for conferring the EETs cardioprotection.

There are several future directions for the work presented in this thesis. One major direction is to assess how EETs modulate the autophagic response as our results did not show how exactly EETs modulated the autophagic flux. Our data suggest that EETs rather stimulate an autophagic response in starved cardiac cells; however, the possibility that EETs might have blocked the autophagic flux cannot be ruled out. Importantly, because there is no one perfect way of measuring the autophagic flux, the use of multiple approaches is essential (89).

It is of particular importance to reproduce these results using different approaches and experimental models. Our group utilizes the Langendorff perfusion system. Although an *in-vivo* model of myocardial IR injury is a more relevant model, *ex-vivo* perfused hearts provide important functional information. Interestingly, it was demonstrated that autophagy represents a protective process following myocardial IR injury in both *in-vivo* and *in-vitro* systems (36, 70, 191). Therefore, it is of great interest at this stage to carry out key experiments in an *in-vivo* model of myocardial IR injury. Our group utilizes two genetically modified mice: *CYP2J2* transgenic mice that overexpress cardiomyocyte-specific *CYP2J2* gene and whole body *sEH* KO mice (*EPXH2*$^{-/-}$), both of which express higher EETs levels. Thus, demonstrating the EET-mediated modulation of an autophagic response in these two models is of great importance. Furthermore, we have recently begun to investigate the cardioprotective effects of EETs in an *in-vivo* model of myocardial IR injury. In these studies, we use *sEH* KO as well as tAUCB-treated WT mice, which undergo left anterior descending coronary artery ligation (LAD) surgery, to assess the EETs cardioprotection. Assessment of the autophagic response in these mice represents an important future direction, and findings of such studies are of great significance because it is a biologically relevant model and various factors are reflected. Taken together, using multiple approaches and experimental models is very important for substantiating our *in-vitro* studies. We anticipate that these future directions will provide novel findings about the EET-mediated protective effects in the myocardium.

APPENDIX

EET-MEDIATED CARDIOPROTECTION AND CAVEOLIN-1

A.1 INTRODUCTION

Caveolae are small vesicles found along the plasma membranes in almost all types of cells. Unlike other lipid rafts, caveolae contain structural proteins. These are cav-1, cav-2 and cav-3. Cav-1 and cav-2 are found in most cell types; however, cav-3 is muscle cell specific (182) Caveolae and caveolins are important in maintaining the CVS homeostasis (132, 133). Although cardiomyopathic phenotypes associated with these mice were recognized several years ago (6, 27, 187), the exact role of caveolae and caveolins in the CVS remains to be determined.

Evidence is accumulating for the involvement of caveolae and caveolins in conferring protection against the myocardial IR injury (79, 86, 131, 133, 173). For instance, it was found that cav-1 is required for IPC-mediated cardioprotection (133). Similarly, Patel et al reported that loss of opioid-mediated cardioprotection following disrupting caveolae (131). Recently, we demonstrated that administration of exogenous EETs or using *Ephx2*$^{-/-}$ mice preserved the caveolar structures and prevented the loss of cav-1 following myocardial IR injury (21). Collectively, these data suggest that caveolae and caveolins are important components of the cardioprotection against the myocardial IR injury.

Autophagy is a highly regulated and conserved process for the digestion of intracellular macromolecules and organelles (90, 197, 198). Amino acid deprivation is a potent activator of autophagy (90, 197, 198).

Evidence suggests that autophagy is robustly induced during the IR injury (40, 71, 110, 175, 191). We have recently reported that activation of autophagy is an important component of the EET-mediated protection in cardiomyocytes (149). Considering it is unknown how caveolae and caveolins are involved in EET-mediated cardioprotection against the myocardial IR injury *i.e.* whether it is a cause or a secondary effect to the EETs protective effects, we sought, in this study, to investigate the involvement of cav-1 in the EET-mediated cardioprotection during cellular starvation.

A.2 MATERIALS AND METHODS

A.2.1 Cell culture and treatment

HL-1 cells (mouse atrial cardiomyocytes) were a kind gift from Dr. Claycomb (New Orleans, USA). Cells were cultivated in Claycomb medium supplemented with glutamine (2mM) and norephinephrine (0.1mM) at 37°C in an atmosphere of 5% CO_2 and 95% air as previously described (26). Neonatal primary cardiomyocytes (NCM) were isolated from the heart of 2-3 days old rat pups as previously described (150). The cells were cultured in DMEM medium supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2 and 95% air. HL-1 cells and NCM were starved in amino acids-free buffer as previously described (149). UA-8 (13-(3-propylureido)tridec-8-enoic acid (1μM), an EET-analogue that possesses EET-mimetic and sEH inhibitory properties (8) was used as an EET model in most experiments. 14,15-EETs was alternatively used in other experiments. 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE, 10μM), a putative antagonist was used to block the EET-mediated effects. HMR-1098 (10 μM) was also used to block the EET-mediated effects.

HL-1 cells were transfected with plasmids that expressing short hairpin RNA (shRNA) against *cav-1* (OriGene Technologies, Rockville, MD). Both Cav-1 targeted shRNA and scrambled negative control (SHAM group) were cloned into a pGFP-V-RS plasmid under a U6 promoter. Plasmids were amplified in *Escherichia coli* (K-12) and purified with

EndoFree plasmid purification kit (Qiagen, Valencia, CA). HL-1 cells were transfected using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen, Burlington, ON, CA). The efficiency of transfection was estimated by the expression of GFP and the efficiency of silencing *cav-1* was estimated by by immunoblotting.

A.2.2 Western blotting

Cells were treated, washed with ice-cold phosphate buffer saline (PBS) and harvested using ice-cold lysis buffer (20 mM Tris-HCl, 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 M sucrose, 1 mM DTT, 1% triton-X100 and protease/phosphatase inhibitors). The cell lysates were incubated on ice for 10 min and centrifuged at 13,000*g* for 15 min (4°C). Total protein contents were measured in the supernatants using Bradford protein assay (BioRad Laboratories, Canada). 20 µg of protein was resolved in 12 or 15% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. Immunoblots were blocked with TBS-T buffer (0.15 M NaCl, 3 mM KCl, 25 mM tris hydroxymethyl methylamine and 0.1% tween-25, pH 7.4) with 5% skim milk for two hours at room temperature and then probed with antibodies to LC3 (Invitrogen, Burlington, ON, CA), cav-1 (Cell Signaling Inc., USA) overnight at 4°C. Relative band intensities to control were expressed in arbitrary units by using Image J software (NIH, USA).

A.2.3 Statistical analysis

Values are expressed as means ± SEM. Statistical significance was determined by using one-way ANOVA followed by Bonferonni post hoc test; values were considered significant when $P < 0.05$.

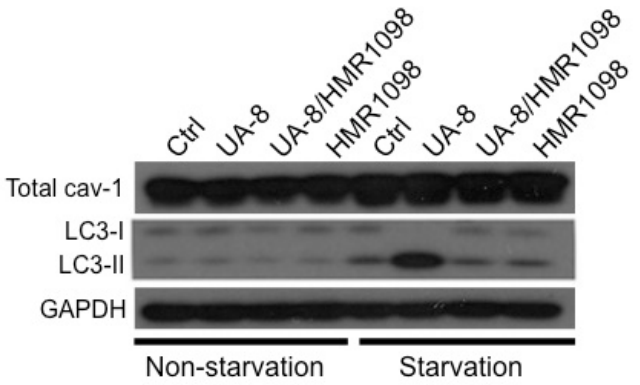
A.3 RESULTS AND DISCUSSION

A.3.1 Effects of starvation and UA-8 treatment on the expression level of cav-1

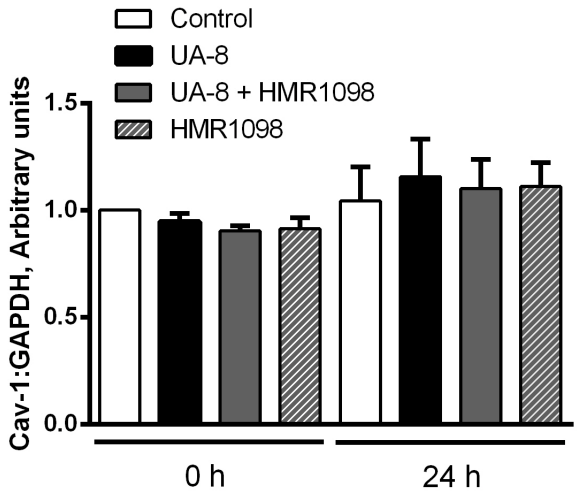
In this part of the study we investigated whether cav-1 expression changes during the EET-mediated modulation of the autophagic response. We sought to answer the following questions: (1) does starvation *per se* affect the expression level of cav-1 in cardiac cells, and (2) does treating cells with UA-8 during starvation affects cav-1 expression in starved cardiac cells. In order to address these questions and similar to studies conducted in the first chapter, we treated NCM with UA-8 (1μM) in the presence or absence of HMR-1098 (10μM). Cells were starved for 24 hours, the point at which EET-mediated modulation of autophagy achieved the peak (149). We found that starvation had no effect on the expression level of cav-1 (Figure A.1A, B). Despite having a pronounced increase in LC3-II expression in cells treated with UA-8 (Figure A.1C), these cells had similar levels of cav-1 expression in comparison to untreated cells. Furthermore, neither HMR-1098 in the presence of UA-8 nor HMR-1098 alone caused any alteration in the expression level of cav-1. Evidence suggests that an increase in the autophagic response is correlated with a decrease in cav-1 expression level in stromal fibroblasts (66, 74, 109, 160, 205). By contrast, it was reported in one study that cav-1 was upregulated by serum starvation in fibroblasts and that restoration of serum caused cav-1 expression to return to basal levels (51). These contradictory

findings could be attributed to different factors such as using different experimental models. It was also reported that starvation caused a downregulation in cav-3 in C2C12 cells, a mouse myoblast cell line (47). One possible explanation for the downregulation of caveolins is that caveolins, as many other proteins, are degraded by the autophagic machinery. Collectively, our findings indicate that starvation-induced increase in autophagy does not affect the expression of cav-1 and thus we concluded that there is no correlation between autophagy and cav-1 expression level in cardiac cells. Furthermore, these results suggest that the EET-mediated modulation of autophagy does not involve alteration in the expression level of cav-1.

A


Ctrl
UA-8
UA-8/HMR1098
HMR1098
Ctrl
UA-8
UA-8/HMR1098
HMR1098
Total cav-1
LC3-I
LC3-II
GAPDH
Non-starvation
Starvation

B


Control
UA-8
UA-8 + HMR1098
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Cav-1:GAPDH, Arbitrary units
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1.0
0.5
0.0
0 h
24 h

C

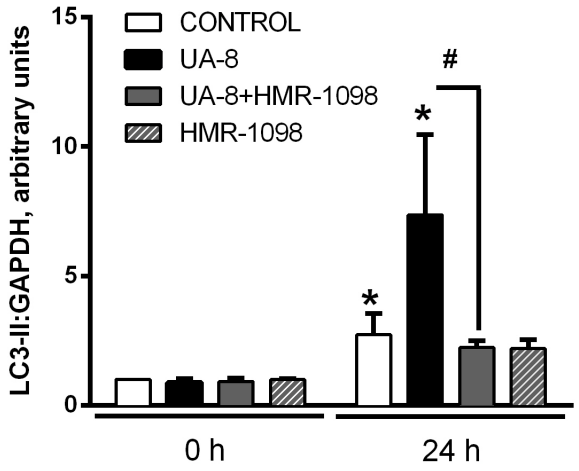


Figure A.1 UA-8-mediated induction of autophagy and the expression level of cav-1

(A) Representative western blots examining the expression level of cav-1 and LC3 under different treatment conditions as indicated. (B) Quantitation of cav-1 shown in A. (C) Quantitation of LC3-II shown in A. Values are represented as mean ± SEM, N=3. Significance was $P<0.05$, *significantly different from control non-starvation, #significantly different from UA-8 treatment.

A.3.2 Effect of silencing cav-1 on UA-8-mediated modulation of the autophagic response

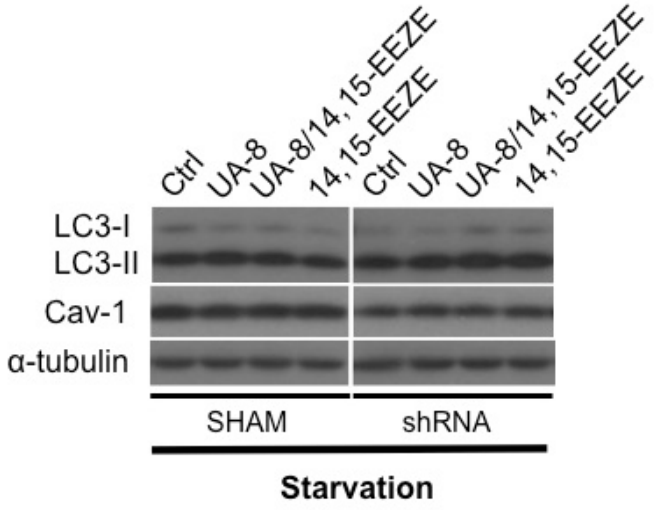
Silencing *cav-1* represents an important approach to study the EET-cav-1 relationship. To address the question whether cav-1 is involved in UA-8-mediated modulation of the autophagic response, we transfected HL-1 cells with short hairpin RNA (shRNA) targeted to *cav-1* or control nonspecific shRNA (SHAM). The efficiency of transfection was estimated by plasmid-tagged green fluorescence protein and the efficiency of silencing the gene was estimated by immunoblotting. Our results showed that the efficiency of the transfection was low *i.e.* limited number of fluorescent cells to the total number of cells, and associated with statistically insignificant difference between the shRNA and SHAM groups at the protein expression level (Figure A.2A, C). This is possibly due to the following reasons: (1) cav-1, as a structural protein, is largely and constitutively expressed with long turnover time; and (2) HL-1 cells, as a type of cardiomyocytes, cannot be effectively transfected even when using different transfection approaches. Therefore, we suggest establishing a stable HL-1 cell line or using viral transfection methods.

Surprisingly, despite having low transfection efficiency, we did not observe the UA-8-mediated modulation of an autophagic response in the cav-$1^{-/-}$ cells. Nevertheless, similar results were seen in the control (SHAM) group *i.e.* loss of the UA-8-mediated modulation of autophagy. This can be attributed to several reasons such as (1) the random effect of

the scrambled gene (SHAM group), or (2) the transfection method by the cationic lipid, Lipofectamine 2000® (Invitrogen). Cationic lipid-based reagents, such as Lipofectamine 2000®, are used to facilitate delivery of nucleic acids into cells (76). This type of reagents, despite having good transfection efficiency in many cells, is known to cause toxicity (76, 122). Lipofectamine 2000® has been shown to induce cellular toxicity at concentration higher than 20 μg/ml (52). Thus, we cannot rule out the possibility that the mechanism of transfection by Lipofectamine 2000® might interfered with caveolae, which found in close proximity, or with the EET-mediated activation of signal transduction pathways. Considering our previous results (21) and the results described in this chapter, we propose that EETs might be involved in redistribution of cav-1 rather than changing the expression level of cav-1. Thus, using other approaches, such as fluorescent-tagged cav-1 and live cell imaging, will better address the temporal and spatial localization of cav-1.

In the current study, we report that the expression level of cav-1 in cardiac cells is not changed during cellular starvation and EETs treatment. Silencing *cav-1* appears to affect the EET-mediated modulation of an autophagic response. However, these results are not conclusive and further experiments are required to confirm these data.

A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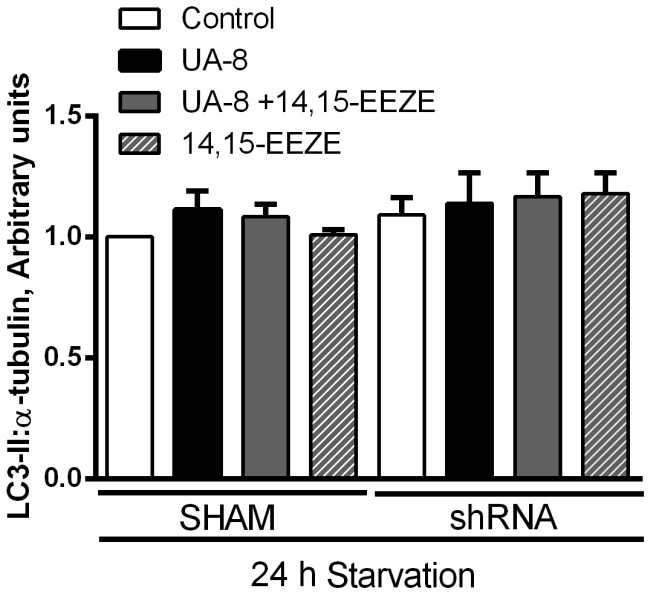
14,15-EEZE
Ctrl
UA-8
UA-8/14,15-EEZE
14,15-EEZE
LC3-I
LC3-II
Cav-1
α-tubulin
SHAM
shRNA
Starvation

B


Control
UA-8
UA-8 +14,15-EEZE
14,15-EEZE
LC3-II:α-tubulin, Arbitrary units
1.5
1.0
0.5
0.0
SHAM
shRNA
24 h Starvation

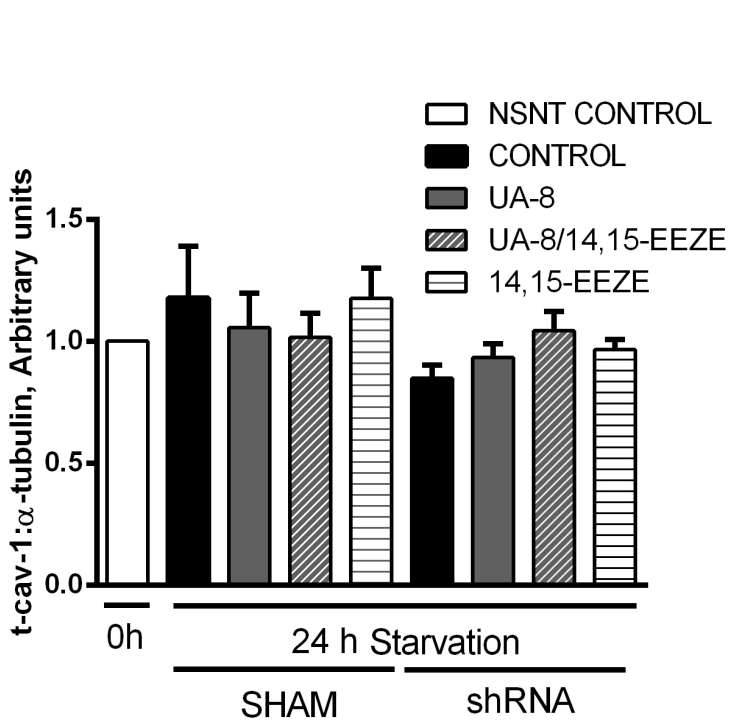


Figure A.2 Silencing cav-1 and the UA-8-mediated induction of autophagic response

(A) Representative western blots examining the UA-8-mediated stimulation of an autophagic response via measuring the expression level of LC3-II under different treatment conditions as indicated. Representative western blot examining the expression level of cav-1 during starvation and under different treatment conditions as indicated (transfection efficiency). (B) Quantitation of cav-1 results shown in A. (C) Quantitation of LC3-II results shown in A. Values are represented as mean ± SEM, N=3. Significance was $P<0.05$.

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