Cardioprotective Properties of a Synthetic, Structurally Mimetic 19,20-Epoxydocosapentaenoic Acid Analog: Implications of SIRT3 Potentiation on Ischemia Reperfusion Injury

By

Joshua Walter Kranrod

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

University of Alberta

ABSTRACT

Mounting evidence suggests that CYP epoxygenase-derived metabolites of docosahexaenoic acid, called epoxydocosapentaenoic acids (EDPs), limit mitochondrial damage following cardiac injury. In particular, the 19,20-EDP regioisomer has demonstrated potent cardioprotective action. Thus, we investigated our novel synthetic 19,20-EDP analog SA-22 for protection against cardiac IR injury. Isolated C57BL/6J mouse hearts were perfused via Langendorff apparatus for 20 minutes to obtain baseline function followed by 30 minutes of global ischemia. Hearts were then treated with either vehicle, 19,20-EDP, SA-22, or SA-22 with the pan-sirtuin inhibitor nicotinamide (NAM), or the SIRT3-selective inhibitor 3-(1H-1,2,3-triazol-4-yl) pyridine (3-TYP) at the start of 40 minutes reperfusion (n = 5-8). We assessed IR injury-induced changes in recovery of myocardial function, using left ventricular developed pressure, systolic and diastolic pressure change. Tissues were assessed for electron transport chain (ETC) function, SIRT-1 and -3, optic atrophy type-1 (OPA1), and caspase-1. We also utilized H9c2 cells and neonatal rat cardiomyocytes (NRCMs) as *in vitro* models of hypoxia/reoxygenation injury. Perfusion with SA-22 significantly enhanced LVDP and dp/dt-max functional recovery (+50 percentage points) compared to vehicle alone, preserved ETC function, SIRT activity, and reduced activation of pyroptosis in response to IR and HR injury. Interestingly, while NAM co-treatment worsened functional outcomes, cell survival, and attenuated sirtuin activity, it failed to completely attenuate SA-22-induced protection against pyroptosis, possibly indicating EDPs exert cytoprotection through pleiotropic mechanisms. In short, these data demonstrate the potential of our novel synthetic 19,20-

EDP analog SA-22 against IR/HR injury and justifies further development of therapeutic agents based upon 19,20-EDP.

PREFACE

This is an original work by Joshua Kranrod. All experimental animal procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and conducted according to strict guidelines provided by the Guide to the Care and Use of Experimental Animals (Vol 1, 2nd ed., 1993, from the Canadian Council on Animal Care). Graphical figures were created using BioRender and GraphPad Prism. Some of the research conducted for this thesis forms part of an international research collaboration, led by Professor J.M. Seubert at the University of Alberta. The main experimental drug studied here (SA-22) was synthesized by Professor J.R. Falck at the University of Texas Southwestern Medical Center with the assistance of Pr A.M. Adebesin and Doctor S. Munnuri. The data analysis and concluding analysis of this thesis are my original work.

At the time of writing, a majority of the contents of this thesis have been previously published as **Joshua W Kranrod**, Ahmed M Darwesh, Wesam Bassiouni, Andy Huang, Liye Fang, Jacob V Korodimas, Adeniyi Michael Adebesin, Sailu Munnuri, John R Falck, and John M Seubert. “Cardioprotective Action of a Novel Synthetic 19,20-EDP Analog is Sirt Dependent” J Cardiovasc Pharmacol. 2024; Jan 1;83(1):105-115. I was responsible for designing and performing the experiments, performing data analysis, and writing the manuscript. Ahmed Darwesh assisted with experimental design and performed Langendorff perfusions. Wesam Bassiouni and Andy Huang performed Langendorff perfusions. Liye Fang assisted with spectrophotometric mitochondria function assessments. Jacob Korodimas assisted with immunoblotting experiments. Adeniyi Michael Adebesin, Sailu Munnuri, and John Falck designed and synthesized the 19,20-

EDP analog SA-22. John M Seubert was the primary investigator and supervisory author who was involved in concept formation as well as review and editing of the manuscript.

Chun / Difficulty at the Beginning

above K'AN THE ABYSMAL, WATER
below CHÊN THE AROUSING, THUNDER

THE JUDGEMENT

Difficulty at the beginning works supreme success,

Furthering through perseverance.

Nothing should be undertaken.

It furthers one to appoint helpers.

THE IMAGE

Clouds and thunder:

The image of Difficulty at the Beginning.

Thus the superior man

Brings order out of confusion

- Hexagram #3, of the I Ching (Richard Wilhelm Translation, Third Edition)

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. John Seubert. You graciously accepted me into your laboratory as a humble summer undergraduate research student over 5 years ago and have continuously provided a wonderful environment for me to grow into the researcher I am today. Your tutelage has been indispensable, and I am truly glad to have completed my MSc program as a member of the Seubert Lab; I would not trade my experiences here for anything in the world!

To my parents, Ken and Paula Kranrod, I am blessed to be your son; I owe everything I have and ever will accomplish to your unconditional love and support. From following you through the forests sampling lichens to publishing my own thesis, I hope this work is something that you can be proud of!

To the members of my supervisory committee, Dr. Michael Doschak and Dr. Tony Kiang, thank you for your invaluable academic insights that have helped make this work the best it could possibly be.

To the predecessor of my project, Dr. Ahmed Darwesh, thank you for starting me off on the right path. Without your past work and advice, this project would not have been possible. I hope you can appreciate where I've built and expanded on your findings.

To Robert Valencia, thank you not just for the fun times and musical jams, but for being my research role model. I attribute much of my own growth to being in proximity to your work. I wish you nothing but good luck during your time at the University of Toronto and beyond.

To my present lab colleagues, Wesam Bassiouni, Mobina Heidary, Liye Fang, Ala' Yousef, and Andy Huang, thank you very much for making the lab a wonderful place to

work. Between your excellent scientific input and quality friendship, I could not have asked for better people to study alongside.

To my past lab colleagues, Jesse Wowk, Hedieh Keshavarz-Bahaghighat, Deanna Sosnowski, and Dr. Kristi Lockhart Jamieson, thank you very much for welcoming me into your group when I was just beginning my research journey. Your mentorship gave me the tools to succeed as a young investigator. While I know you don't need it, I wish you all good luck in your future endeavours.

To Dr. Kousei Ito, Dr. Shigeki Aoki, Zhiheng Zhang, and all other members of the Laboratory of Biopharmaceutics at the University of Chiba, I thank you from the bottom of my heart for welcoming me into your group and making me feel at home during my time in Japan. While the contents of our collaboration did not make their way into this work, I feel that my experiences with your lab were an invaluable part of my MSc program, so for that I thank you. I hope your relationship with the Seubert Lab remains for fruitful for many years to come.

Thank you to everyone in the Faculty of Graduate Studies and Research as well as the Faculty of Pharmacy and Pharmaceutical Sciences at the University of Alberta for your financial assistance and support during my time here.

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3-TYP	3-(1H-1,2,3-triazol-4-yl) pyridine
AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
AceCS2	Acetylcoenzyme A synthase 2
ACK	Acetylated lysine
ADP	Adenosine diphosphate
ALA	α-Linoleic acid
AMC	7-Amino-4-methylcoumarin
AMI	Acute myocardial infarction
AMPK	5’ Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ANT1	Adenine nucleotide translocator
ATCC	American type culture collection
ATP	Adenosine triphosphate
BA	Beta-actin
BME	2-Mercaptoethanol
BPM	Beats per minute
BSA	Bovine serum albumin
CBR	Cannabinoid receptor
CCR	Coupling-control ratio
CHD	Coronary heart disease
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate

CMV	Cytomegalovirus
CLV	Cleaved
CoQ	Coenzyme Q_{10}
COX	Cyclooxygenase
COX IV	Cytochrome c oxidase subunit 4
CS	Citrate synthase
cTnT	Cardiac troponin t
CVD	Cardiovascular disease
CYP	Cytochrome p450
CZI	Carl Zeiss Image
DAMP	Danger-associated molecular pattern
DCPIP	2,6-Dichlorophenolindophenol
ddH_2O	Double-distilled water
DHA	Docosahexaenoic acid
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethylsulfoxide
D-PBS	Dulbecco’s phosphate-buffered saline
DRP-1	Dynamin-related protein-1
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
DUB	Decylubiquinone
EDP	Epoxydocosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid

EEQ	Epoxyeicosatetraenoic acid
EET	Epoxyeicosatrienoic acid
EPA	Eicosapentaenoic acid
EpFA	Epoxy fatty acid
ET	Electron transport capacity
ETC	Electron transport chain
FA	Fatty acid
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FCE	Flux control efficiency
FCR	Flux control ratio
FFAR	Free fatty acid receptor
FL	Full-length
Foxo	Family of forkhead transcription factors
FPR	Formylated protein receptor
GFP	Green fluorescent protein
GOE	Great oxidation event
GPCR	G protein-coupled receptor
GSDMD	Gasdermin-D
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HEPE	Hydroxyeicosapentaenoic acid

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Heart failure
HIF-1α	Hypoxia-inducible factor-1α
HR	Hypoxia-reoxygenation
HRP	Horseradish peroxidase
hSIRT3	Human sirtuin-3
HSP	Heat shock protein
IMM	Inner mitochondrial membrane
IL-1β	Interleukin 1 beta
IPC	Ischemic pre-conditioning
IR	Ischemia-reperfusion
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
LCAD	Long-chain acyl-CoA dehydrogenase
LEAK / $_{L}$	Leak respiration / State 4 / ATP-independent respiration
LKB1	Liver kinase B1
LP2K	Lipofectamine 2000
LPS	Lipopolysaccharide
LVDP	Left ventricular developed pressure
LYS	Lysine
MoA	Mechanism of Action
MiR05	Mitochondrial respiration medium 05
$MnSOD_2$	Manganese superoxide dismutase
mPTP	Mitochondria permeability transition pore

mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hydrogen
NAM	Nicotinamide
NFP	Natural formylated protein
NLRP3	Nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain containing 3
NRCM	Neonatal rat cardiomyocytes
NRF2	Nuclear factor erythroid 2-related factor 2
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy type 1
OXPHOS / P	Oxidative phosphorylation respiration / State 3 / ATP-dependent respiration
P2X7R	Purinergic receptor X subtype 7
P62	Sequestosome-1
PARP1	Poly-ADP-ribose polymerase 1
PBS	Phosphate-buffered saline
PCR	Pathway control ratio
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PI3K	Phosphoinositide 3-kinase
PINK1	Phosphatase and tensin homolog (PTEN)-induced kinase 1

PLA2	Phospholipase A2
PM	Pyruvate + Malate
pmF	Proton motive force
PMG	Pyruvate + Malate + Glutamate
PMGS	Pyruvate + Malate + Glutamate + Succinate
PMN	Polymorphonuclear leukocyte
PPAR	Peroxisome proliferator-activated receptor
PPI	Protease and Phosphatase inhibitors
PTM	Post-translational modification
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene difluoride
RA	Retinioic acid
RC	Respiratory chain
RCR	Respiratory control ratio
RLU	Relative light unit
ROS	Reactive oxygen species
ROX	Residual oxygen consumption
RPM	Revolutions per minute
SA-22	(10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid
SDH-A	Succinate dehydrogenase subunit A
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sEH	Soluble epoxide hydrolase

SEM	Standard error of mean
SER	Serine
SFA	Saturated fatty acid
SIRT	Sirtuin / silent mating type information regulation 2 homolog
SRC	Spare respiratory capacity
SUIT	Substrate-uncoupler-inhibitor titration
tBID	BH3 interacting-domain death agonist
TBST	Tris-buffered saline tween
TCA	Tricarboxylic acid cycle
TFA	*Trans* fatty acid
TFAM	Mitochondrial transcription factor A
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TORC2	Target of rapamycin complex-2
VDAC	Voltage-dependent anion channel
VSV	Vesicular stomatitis virus
YME1L1	Nuclear encoded mitochondrial escape 1-like

CHAPTER 1. INTRODUCTION

1.1 Ischemic heart disease and reperfusion injury

Morbidity and mortality due to ischemic heart disease (IHD) continues to be a major global health issue(1,2). The Canadian Cardiovascular Society reports that cardiovascular disease (CVD) accounts for nearly a third of all death(3). According to the Public Health Agency of Canada, an estimated 8.5% of Canadian adults (>20yrs) live with diagnosed IHD. With over 60,000 Canadians having their first heart attack every year, the clinical burden of IHD is immense(4). The Canadian Heart Health Strategy and Action Plan Steering Committee estimates that the yearly costs associated with CVD (i.e. hospitalizations, physician services, lost productivity, etc.) total more than $22.2 billion(5). As such, the development of clinical strategies for the treatment/prevention of IHD is highly desirable.

IHD is a subtype of CVD caused by the blockage or narrowing of coronary arteries that supply the myocardium with oxygenated blood vital for tissue function and survival, thus subjecting its constitutive cells to ischemia(6). While blood vessel narrowing can be caused by a variety of factors including vasoconstriction or blood clotting, the primary etiological factor is plaque buildup, also known as atherosclerosis(6,7). IHD has a multitude of risk factors/comorbidities that vary in significance including age, sex, diet, lifestyle, and genetics(8). Despite extensive research, the gold standard treatment for ischemia remains the immediate restoration of myocardial blood-flow(9-11). Paradoxically, reperfusion triggers a secondary wave of myocardial cell death known as ischemia-reperfusion (IR) injury(12,13). As cardiomyocytes have a limited regenerative capacity, the myocardium is especially vulnerable to IR injury(14,15). The lack of a definitive, singular mechanism by which cell death is mediated following ischemia-

reperfusion has confounded the development of efficacious therapeutic agents thus far. Traditional thinking assumes that during ischemia, prevention of coronary blood-flow deprives cardiomyocytes of oxygen, a critical component for oxidative phosphorylation (OXPHOS). The resulting metabolic shift towards anaerobic glycolysis subsequently lowers cytosolic pH and causes an accumulation of intracellular calcium. Upon restoration of physiological pH, calcium triggers mitochondria depolarization via the mitochondrial permeability transition pore (mPTP) (16-18). Opening of the mPTP induces mitochondrial swelling and membrane rupture, causing the release of cytochrome c and subsequent activation of programmed cell death pathways(19). However, post-IR cytosolic release of cytochrome c following mitochondrial depolarization independent of mPTP opening has also been observed, suggesting the involvement of multiple signaling pathways in the pathogenesis of IR injury(19). Subsequently, modern cardiovascular research has begun establishing links between mitochondrial injury/dysfunction, innate immune responses, and IR-induced necrotic myocardial cell death.

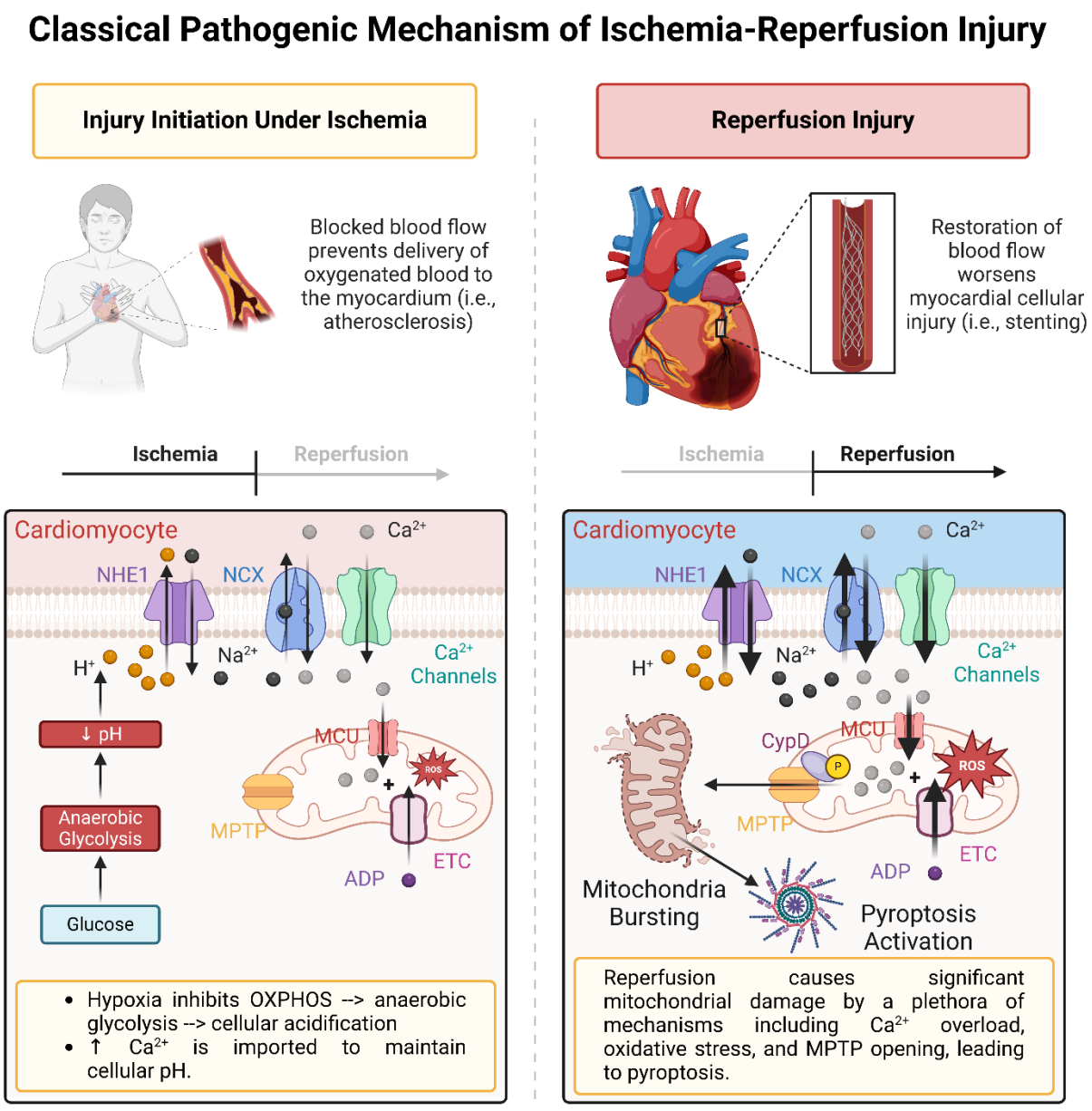


Figure 1.1. *Graphical summary of classical ischemia-reperfusion injury pathogenesis.*

1.2 Previous therapeutic approaches to IR injury and obstacles for clinical translatability

For over 50 years it has been appreciated that the magnitude of myocardial tissue damage following coronary occlusion is not determined immediately at the onset of ischemia but can be altered by therapeutic interventions applied during ischemia(20). From this moment onwards cardiologists have sought to find the 'Holy Grail', a prophylactic therapy for IHD. However, despite the identification of several hundred cardioprotective interventions (both pharmacologic and nonpharmacologic) in experimental animal models, none besides timely reperfusion has been translated into clinical practice(21).

Perhaps the most infamous example of this is known as ischemic preconditioning (IPC). First discovered in 1986, IPC describes a phenomenon wherein tissues subjected to transient periods of ischemia become resistant to subsequent sustained ischemic insults(22). IPC was a source of great optimism in the 1990s field of cardiology for it consistently reduced infarct size across all experimental animal models(10). Unfortunately, IPC is faced with an unreconcilable translational problem in that the onset of MI in humans is highly unpredictable. Despite promising subsequent studies finding that applying intermittent coronary occlusions post-ischemia, at reperfusion, or in remote vascular beds (i.e., brachial artery) pre-reperfusion (known as "remote conditioning") could limit infarct size, these approaches all failed to demonstrate efficacy in larger, randomized and controlled clinical trials(23-26).

Beyond IPC, numerous interventions have exhibited cardioprotective properties in experimental animal models only to return negative results in larger clinical trials. The

enzyme hyaluronidase was theorized to protect the heart against acute myocardial ischemia by reducing tissue edema as a result of depolymerizing hyaluronic acid, a major mucopolysaccharide constituent of the cardiac interstitum, subsequently enabling proper diffusion of nutrients and metabolic waste(27,28). However, the efficacy of hyaluronidase treatment was inconsistent in experimental studies and did not translate when examined clinically(29,30).

The angiotensin-converting enzyme (ACE) inhibitor captopril initially held promise as an adjuvant to thrombolytic therapy for acute myocardial infarction (AMI). It was believed that the ability of captopril to scavenge free radicals, stimulate vasodilation, and blunt catecholamine signaling would ameliorate tissue damage following infarction. Unfortunately, captopril appeared to merely delay ischemic events following cardiac surgery rather than prevent them(31).

The surfactant polymer poloxamer-188 possesses antithrombotic and hemorheological properties that made it attractive for potential use as part of reperfusion strategies. Again, while initial experimental studies suggested it is effective at reducing myocardial tissue damage when used alongside thrombolytic therapy, more substantial clinical trials yielded no significant efficacy(32,33).

Infamous for its involvement in various international athletic doping scandals, trimetazidine was initially developed to treat agina pectoris(34,35). Subsequent experimental studies seemed to conclude that trimetazidine can reduce ischemic oxidative stress by inhibiting fatty acid oxidation (FAO), consequently promoting more efficient glucose oxidation(36). In contrast, the European Myocardial Infarction Project – Free Radicals (EMIP-FR) group found that trimetazidine had no significant benefit with

respect to short- and long-term outcomes of patients following AMI, regardless of whether they received thrombolytic therapy. In fact, trimetazidine appeared to worsen the incidence of short-term deaths following ischemia when compared to placebo(37).

It is clear from the multitude of translational failures the field has experienced that many barriers obfuscate the clinical applicability of most therapeutic approaches developed in experimental models. Naturally, it is to be expected that irreproducible pre-clinical results will not result in successful clinical outcomes. Experimental studies frequently suffer from several factors including a lack of standardized models, protocols, and methods of analysis, non-randomized study design and/or lack of investigator blinding, methodological errors, and establishment of meaningful endpoints. For instance, many studies report cardioprotective effects despite treating pre-ischemically. Such findings are almost entirely irrelevant given the unpredictability of heart attacks. Furthermore, care must be taken when considering how well a model approximates a clinical setting. While the use of isolated hearts and cardiomyocytes from young, healthy animals provides valuable mechanistic insight, these models often underestimate the severity of real-world myocardial ischemia. For one, young animals typically lack many of the comorbidities associated with adverse cardiac outcomes(38,39). Isolated tissue and culture systems also lack multifactorial and -tissue interactions that regulate *in vivo* injury responses. A lack of appropriate experimental endpoints has contributed to the difficulty in development therapies for IR injury. For instance, traditional thought has approximated tissue infarct size as a surrogate for overall tissue injury. However, it is entirely unknown whether infarct size is linearly related to the incidence of adverse cardiac outcomes. Additionally, it has even been observed that reducing AMI incidence may not be sufficient

for reducing clinical complications(40). Furthermore, significant limitations facing the imaging of cardiac injury and infarction have made the dubious accuracy of current assessment methods a point of contention(21). While this study observes the potentially cardioprotective properties of a synthetic epoxy fatty acid (EpFA) drug, we acknowledge that extensive work and collaboration is required to reasonably propose clinical consideration of such compounds.

1.3 Pyroptosis and mitochondrial DAMPs

Growing evidence indicates that pyroptosis, a form of programmed inflammatory cell death, plays a central role in the pathogenesis of myocardial IR injury(41-43). Strong cellular stressors and/or injury such as ischemia and lipopolysaccharide (LPS) induce the intracellular release of danger-associated molecular patterns (DAMPs) (i.e. oxidized mtDNA), pro-pyroptotic signals that stimulate the proteolytic cleavage and activation of the caspase-1/ gasdermin D (GSDMD)/ interleukin-1 beta (IL-1β) signaling pathway, ultimately resulting in pyroptotic cell death(44,45). Briefly, DAMPs are recognized by various ligand-specific receptors that activate inflammasomes, macromolecular structures which transduce pathogenic signals into innate immune responses(46,47). Importantly, DAMPs are endogenous molecules which are capable of activating inflammasomes independent of any pathogen/injury, meaning that inflammasomes and pyroptosis are likely to be involved in cell death arising from rapidly-developing forms of cellular injury such as post-ischemia-reperfusion injury(48,49). Other groups have already demonstrated that targeting pyroptosis is a viable target for amelioration of IR injury(50-53). Furthermore, prior studies have shown that maintenance of sirtuin activity also holds promise as a novel approach for blunting

inflammatory cell death(54-58). Additionally, literature indicates that mitochondrial dysfunction heavily contributes to adverse cardiac outcomes following IR injury, as approaches to preserve mitochondrial integrity limit cardiac damage(12,59). While they are the main provider of energy driving myocardial contractility, mitochondria are also a significant source of inflammasome-activating DAMPs(60). Known mitochondrial DAMPs include mitochondrial DNA (mtDNA), mitochondrial transcription factor A (TFAM), ATP, Succinate, Cardiolipin, and N-formyl peptides(61). Interestingly, despite the vital roles some of these molecules have with respect to cellular metabolism and survival, elevating their cytosolic concentration often triggers cell death.

Recent studies have demonstrated that mtDNA is a strong inducer of pyroptotic cell death mediated thru the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome(62). Importantly, NLRP3 is well-known for its role in transducing pathogenic signals into activation of caspase-1 in a variety of injury models, including IR injury(49,53,63). Furthermore, NLRP3 is a known inhibitor of autophagy, a vital intracellular process by which damaged and/or dysfunctional proteins and organelles are removed and degraded(64). Inhibition of autophagy is known to result in the accumulation of dysfunctional mitochondria, ROS, and oxidized mtDNA, subsequently aggravating caspase-1 activation(62). Notably, it has been shown that mice deficient in autophagy proteins are more vulnerable to septic shock and cardiac failure, injuries associated with DAMP-mediated inflammation(62,65,66). Additionally, mtDNA is known to stimulate inflammatory interferon production via activation of the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING)-mediated type I pathway(67-69). While the deleterious

effects of cytosolic mtDNA release have been well documented, the mechanisms regulating its release are still poorly understood.

The primary source of energy in all living organisms, ATP also functions as a tightly regulated signaling molecule. ATP serves to propagate pyroptotic death on neighboring cells following cellular lysis. Extracellular ATP binds and activates the purinergic receptor X subtype 7(P2X7R), causing rapid potassium efflux and subsequent caspase-1 activation(70,71).

Known to play essential roles with regards to mtDNA copy number, maintenance and transcription, the HMG box family member TFAM is also a mitochondrial DAMP(72,73). While myocardial TFAM deficiency is associated with severe respiratory dysfunction, liberated TFAM is known to be pro-inflammatory(74,75). Notably, exogenous addition of recombinant TFAM increases tumor necrosis factor (TNF) and IL-6 expression in both rats and RAW264.7 macrophages(76).

Following induction of hypoxia-mediated necrosis, natural formylated peptides (NFPs) are derived from human mitochondrial proteins(77). Expressed exclusively by necrotic cells, formylated proteins serve as low-molecular-weight chemoattracts for phagocytes, where they are recognized by formyl peptide receptors (FPRs) (78,79). FPRs are members of the seven transmembrane G protein-coupled receptor (GPCR) superfamily and upon ligation of NFPs, allow the influx of Ca^{2+}(77). NFPs have a plethora of immunomodulating properties including the generation of ROS intermediates, stimulation of phagocytosis, and release of proteolytic enzymes(80). Interestingly, treatment with both purified NFPs and synthetic NFP analogs stimulates

human polymorphonuclear leukocyte (PMN) chemotaxis *in vitro*(79,81). Additionally, NFP signaling synergizes with the pro-inflammatory effects of cytosolic mtDNA(60).

Under normal physiological conditions, succinate functions as an essential electron donor for the ETC(82). Synthesized as part of the mitochondrial TCA cycle, it has been demonstrated that succinate can be secreted to the extracellular space *in vitro*(83). Secretion of succinate appears to be increased upon the induction of necrosis(83). Notably, extracellular succinate has been shown to trigger intracellular calcium mobilization and enhance TLR-induced inflammatory cytokine production(84). Furthermore, succinate promotes innate immune responses, marked by the expression of IL-1β(85). Interestingly, LPS (a known inducer of pyroptosis), increases the glycolytic production of succinate(85). The broader literature also indicates that succinate plays a role in the pathogenesis of several CVDs including pulmonary artery hypertension and myocardial hypertrophy(86).

Comprised of two phosphatidyl groups connected by glycerol, cardiolipin is a critical regulator of mitochondrial function including respiration and biogenesis(87,88). Experimental data correlates cardiolipin dysregulation with the pathogenesis of several diseases(89). Furthermore, cardiolipin appears to have several roles involved with pyroptotic cell death including MOM permeabilization, cytochrome c release, and direct NLRP3 inflammasome activation(90,91). Along with all other mitochondrial DAMPs, cardiolipin is implicated in a wide range of human diseases(61). Thus, if not just for the maintenance of bioenergetic homeostasis, preventing the cytosolic release of DAMPs by protecting mitochondrial health is crucial for ameliorating IR injury.

1.4 Mitochondria homeostasis and IR injury pathogenesis

Uniquely positioned as the organ responsible for delivering blood to the entire body, the heart has immense energy demands for the maintenance of physiological homeostasis(92,93). To meet these demands, the myocardium possesses an extensive number of mitochondria, the organelles responsible for producing energy substrates that enable/drive tissue contractility(92-94). Unfortunately, being so metabolically active confers sensitivity to IR injury unto the myocardium, as disruptions to mitochondrial metabolism quickly result in depletion of intracellular ATP and accumulation of mitochondrial ROS (mtROS), subsequently leading to oxidative damage of mtDNA, proteins and many other cellular constituents (95). In fact, mitochondrial damage is considered to play a key role in the progression from myocardial IR injury into chronic heart failure(96). Thus, the development of new pharmacological agents capable of galvanizing mitochondrial health to minimize the cardiovascular dysfunction resulting from IR injury is tantalizing.

1.5 Endosymbiotic theory of mitochondria

Arising from the Greek *endo* meaning ‘within’ or ‘containing’ and *symbiosis* meaning ‘together’ and ‘living’, the term endosymbiosis refers to the idea that biological organisms can exist as composite beings made up of multiple discrete organisms(97). The origins of modern endosymbiotic theory emerged in the nineteenth century due to difficulties in phyletically classifying lichens. In 1867, the Swiss biologist Simon Schwendener was the first to propose that lichens were composite organisms, constituted of both fungi and alga(98). In the contemporary scientific context, endosymbiosis most

commonly refers to the concept of divergent evolutionary ancestry regarding mitochondria; that they arose from primordial α-protobacteria, separate from their eukaryotic host cells(99). While it would gain modest support from a handful of other biologists in the later nineteenth century, it would not be until the advent of genetic sequencing and the work of Lynn Margulis in the mid-twentieth century that the endosymbiotic origin of mitochondria began to achieve widespread acceptance(100-104). While the reasons behind why and how the symbiosis between eukaryotes and mitochondrion came to occur remain unspecified or controversial, it is widely accepted that mitochondrial bioenergetic capacity is supremely vital for eukaryotic genome complexity and evolution(105,106). It has been estimated that genetic restructuring due to mitochondrial endosymbiosis enabled a 200,000-fold increase in gene expression when compared to prokaryotes(107). Furthermore, the virtually complete absence of a 'missing link' organism bridging the evolutionary gap between modern day and pre-endosymbiotic organisms continues to confound investigators(99). It is important to remember that prior to the advent of photosynthetic metabolism by cyanobacteria and *Prochlorococcus*, Earth's atmosphere was virtually anoxic(108). Prior to endosymbiosis, mitochondrion likely relied upon anaerobic respiration for survival and were therefore susceptible to oxygen toxicity. A traditional school of thought holds that the engulfing of mitochondrion by prototypical eukaryotes was a mutually beneficial adaption to the Great Oxidation Event (GOE) caused by photosynthetic protists(109-112). However, it should be noted that this conclusion is disputed by estimates stating that the last common ancestor of eukaryotes (born of mitochondrial endosymbiosis) likely arose millions of years after the GOE(113). Regardless, the centrality of mitochondrial homeostasis with

respect to hypoxic injury should be appreciated not just in the context of acute injury, but that of evolutionary adaptation.

1.6 Mitochondrial respiration and oxidative phosphorylation

Published over 60 years ago, Peter Mitchell's theory of chemiosmotic coupling provided a general explanation for photosynthetic, microbial, and mitochondrial energy transformation(114). His work gave credence to prior conceptualization of human cells as a symbiotic 'supraorganism' within which discrete organelles, granula, and other microorganisms could contribute to cooperative metabolic pathways as described by Richard Altmann(115). Known as the transmembrane protonmotive force *pmF*, chemiosmotic coupling ultimately attempts to relate H^+ translocation to electron transfer and subsequent production of ATP from ADP(116). Two main forces comprise the *pmF*, a diffusive component ($\Delta_d F_{H+}$) related to the change in H^+ concentration across a membrane, and an electric component ($\Delta_{el} F_{p+}$) which is equivalent to mitochondrial membrane potential ($\Delta\Psi$). Thus, mitochondrial respiration can be summarized as the "exergonic input of catabolism coupled to the endergonic output of phosphorylation" (117). Taken further, Mitchell's theory of chemiosmotic coupling is contextualized with the following points(118):

a) Electron transfer chain components are arranged vectorially across the mitochondrial membrane such that electron transfer is linked to proton translocation across the membrane.
b) The mitochondrial membrane forms a tightly closed structure which is impermeable with respect to H^+.

c) H^+ may return only through ATPase, whereupon they are utilized in ATP synthesis. The mitochondrial membrane also possesses additional proton-linked transporters necessary for osmotic stabilization and metabolite transport.

To achieve a sufficient transmembrane electrochemical potential difference (or *pmF*) necessary for driving ATP synthesis/OXPHOS, charge separation across the mitochondrial membrane is required. Generating this electrochemical gradient is the collective responsibility of the four distinct supramolecular respiratory ETC protein complexes(116): NADH:ubiquinone oxidoreductase (complex I), succinate dehydrogenase (SDH, complex II), decyl-ubiquinol cytochrome C oxidoreductase (complex III), cytochrome C oxidase (complex IV). While Mitchell's theory of chemiosmotic coupling recognized that electron transfer was intrinsically tied to proton translocation, a novel revolutionary concept for the time, it was somewhat limited in its ability to explain how ETC components went about accomplishing this, as the prevailing view of the mitochondria at that time was that of a diffuse 'bag-of-enzymes'(119). The Singer-Nicholson model assumed that electron transfer was dependent upon random collisions between complexes I-IV and/or CoQ(120). Fortunately, subsequent works and discoveries have elucidated how OXPHOS is coordinated across the ETC. Briefly, complexes I and II oxidize NADH and succinate respectively, and simultaneously reduce ubiquinone (coenzyme Q_{10}, CoQ), forming ubiquinol(121,122). Ubiquinol then shuttles electrons to complex III(123). Complex III then catalyzes the transfer of electrons from ubiquinol to cytochrome c(124). Complex IV first oxidizes cytochrome c then reduces molecular oxygen(125). The *pmF* generated as a consequence of proton trans-localization due to complex-mediated electron transfer then triggers a conformational

change in ATP synthase that forces ADP and phosphate together, resulting in ATP synthesis(126). Effluxing protons simultaneously form water with the oxygen that was oxidized by complex IV. Importantly, a relatively small number of protons are sufficient to both saturate mitochondrial membrane capacitance (i.e. charge the membrane) and generate significant water column hydrostatic pressure; Both of which are helpful in performing chemical work. This relative ease of access to energetic work potential enabled by ATP synthase means that OXPHOS generates significantly more ATP with less metabolic waste than other forms of respiration(127,128).

That is not to say that the OXPHOS and ETC are without flaw; While achieving charge separation enables the creation of ATP synthesis in a biological system that is electrically uncharged at macro, this also positions the ETC as the pre-eminent source of cellular ROS(129). When describing the theory behind the translation of NADH/FADH oxidation into the production of ATP, we assume that electrons are transferred perfectly or ‘coupled’ between components of the ETC. Unfortunately, this is almost never the case, as electron ‘leak’ due to various issues such as steric interference, membrane disruption or complex dysfunction. Electrons frequently leak out from the mitochondrial matrix and interact with oxygen, subsequently forming reactive superoxides(130,131). Reports have indicated the existence of eleven sites which significantly contribute to the production of ROS by the ETC(132). ROS generated by complex I appears to arise mainly from sites that are involved with binding CoQ(133). Evidence indicates that complex II-mediated ROS production relies upon the proximity of oxygen to reduced flavoprotein at site II_F, as molecules which block oxygen binding inhibit the production of ROS(82,122,132). ROS derived from complex III appears to be a result of accidental reactions between electron-

carrying ubisemiquinone travelling within the matrix-facing Q-junction and molecular oxygen diffusing across the mitochondrial membrane(134-136). Structural features present within complex IV appear to minimize the generation of ROS(137).

While ROS have been demonstrated to have important physiological roles as signaling molecules, these functions are mainly relevant at basal levels and should not confound well-established understanding regarding ROS as stimulators/mediators of cell death(129,138,139). Briefly, significant ROS production can cause lipid peroxidation, impede mitochondrial respiration and ATP synthesis, and oxidize proteins as well as DNA, often culminating in cell death(140). Previous experimental studies have shown that limiting the accumulation of oxidative species by treating with antioxidants can preserve myocardial function despite IR insult(141,142). Importantly, it has been demonstrated that ETC-derived ROS plays a significant role in cardiac IR injury(143). Furthermore, pathological levels of ROS have been associated with a plethora of CVDs including atherosclerosis, cardiac hypertrophy, cardiomyopathy, and heart failure(144-147). Thus, if not simply for preventing the cytosolic release of mitochondrial DAMPs, protecting mitochondrial homeostasis against IR injury is important for limiting ROS production and subsequent induction of cell death signaling pathways.

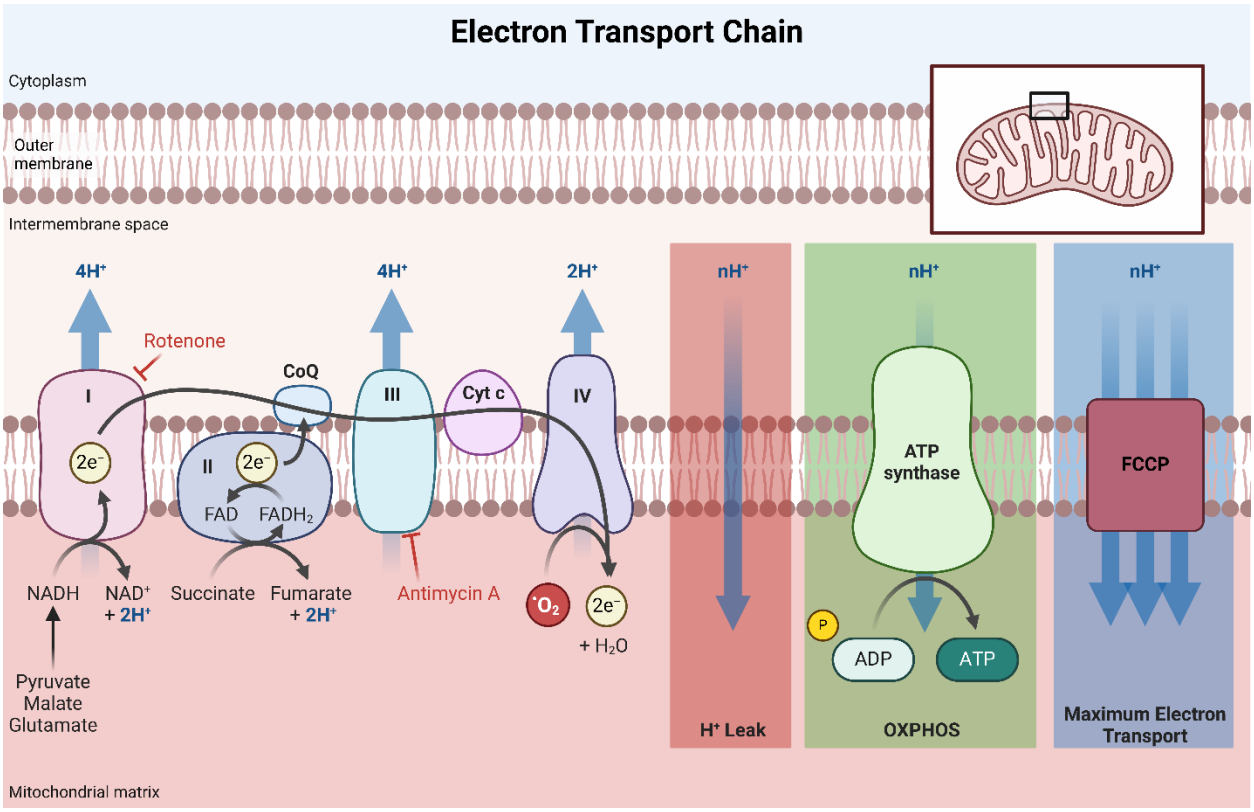


Figure 1.2. *Graphical depiction of the mitochondrial electron transport chain*.

1.7 ETC supercomplexes and IR injury

While the assembly of respiratory chain (RC) complexes into super-complexes was theorized over 60 years ago, the functional implications of this were not extensively explored until relatively recently(148,149). Research into RC super-complexes at the time was hindered by significant methodological limitations and a prevailing view of the ETC as a diffuse 'bag-of-enzymes' as described by the Singer-Nicholson model(120,150). Now dubbed 'respirasomes', RC super-complexes and their physiological roles in disease pathogenesis have become a hot topic for researchers(151-153). In particular, a respirasome comprised of complexes I, III, and IV is now understood to be the pre-eminent super-complex in mammalian mitochondria(154). Significant for increased

respiratory efficiency and decreased ROS production attributable to proton leak, experimental data indicates that respirasome assembly plays a significant role in cellular resistance to injury/stress(155-160). Interestingly, respirasome assembly appears to be affected/diminished by post-translational modifications (PTMs) (including acetylation) of its constituent ETC complexes(161,162). Additionally, evidence suggests that the benefits of respirasome assembly with respect to ROS production are attributable to minimizing the distance between electron transferring iron-sulfur clusters present with the various ETC complexes(163). This is unsurprising given the well-documented chemical tendency of iron to readily produce superoxides via Fenton reactions, and its biological significance with regards to ferroptosis, a form of oxidative cell death characterized by rampant ROS production caused by iron ions(164-166). Recent evidence indicates that mitochondrial iron-derived ROS is central to the pathogenesis of myocardial infarction(167-169). In this study we correlate changes in mitochondrial acetylation with changes in respiratory activity.

1.8 Association of PUFAs with cardioprotection

N-3 polyunsaturated fatty acids (PUFAs) are abundant in the human body and serve both as critical components of phospholipid membranes and as precursors to a wide variety of eicosanoids(170,171). Major dietary sources for n-3 PUFAs include fish, eggs, bread, canola oil, soybean oil, flaxseed nuts, and leafy greens(172). The primary n-3 PUFAs provided by these foods are α-Linoleic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA). PUFAs are primarily metabolized through three distinct enzymatic pathways, cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) enzymes to a plethora of bioactive lipid mediators(171,173).

While ALA is capable of being enzymatically converted into DHA and EPA, this activity is fairly limited in humans(174). It has long been recognized there exists a positive relationship between dietary intake of fatty acids and a reduction in populational cardiovascular disease (CVD) (175-177). One of the first such observations came when a group speculated that the lower mortality attributable to CVD among Greenland Inuits could be due their elevated dietary fish oil consumption(178). Further study of populations with diets similarly rich in n-3 PUFAs in Nunavik and Japan seem to support these claims(179). The “GISSI-HF Trial”, a randomized, double-blind, placebo-controlled trial demonstrated that n-3 PUFA treatment could help ameliorate patient mortality and hospitalization following heart failure (HF) (180). A plethora of groups have also observed that PUFAs lower serum cholesterol levels, subsequently reducing the risk of coronary heart disease (CHD) (181-183). Notably, the protective effects of PUFAs contrast with other fatty acids (i.e., *trans* fatty acids (TFAs) and saturated fatty acids (SFAs)) (184-186). Later studies would go on to demonstrate that the presence of PUFAs could be associated with reduced cardiac arrythmias, myocardial infarction, plasma triglycerides, ventricular fibrillation, blood viscosity, and blood pressure(184,185,187,188). It is important to maintain proper discretion when considering n-3 PUFAs as potential therapeutics for cardiovascular diseases however, as several groups have also observed that n-3 PUFAs may exert little to no beneficial effect in the context of several different cardiac injuries(189-191). Furthermore, extensive physiological and genetic adaptation may be required for the human body to take full advantage of a diet rich with n-3 PUFAs(192). Despite the controversy surrounding whether n-3 PUFAs can prevent CVDs, a wealth of experimental studies have demonstrated cardioprotective benefits associated

with n-3 PUFA intake and consider them to be vital regulators of myocardial health(193-198).

1.9 Cytochrome P450s and PUFA metabolism

More than 6000 distinct monooxygenases comprise the CYP enzyme super-family(199). Subsequently, CYP enzymes have established roles in the metabolism of a wide range of lipophilic molecules that includes xenobiotics, fatty acids, biogenic amines, vitamins, and steroids(199). Traditionally associated primarily with hepatic metabolism, increasing evidence indicates that CYP enzymes also have important extrahepatic functions(200,201). PUFAs, readily metabolized into EpFA metabolites by CYPs(202,203), are often shared/transformed by multiple different enzyme isoforms. Notably, many CYP isozymes exert unique stereo- or regioselectivity toward specific substrates, including PUFAs. The regulation of PUFA/EpFA species by CYP-mediated metabolism is further complicated by tissue- and sex-specific factors such as hormones, transcription- and growth factors(204,205). Among mammalian cells, CYP enzymes exhibit limited mitochondrial localization, instead being found more commonly within the endoplasmic reticulum(206). With respect to tissue-specific expression, CYPs can be found in many tissues throughout the body, including the lungs, heart, gastrointestinal tract, kidney, and brain(204,205,207,208). The mammalian myocardium expresses a plethora of CYP isozymes including, CYP1A, CYP1B, CYP2A, CYP2B, CYP2D, CYP2E, CYP2J, CYP2R, CYP2S, CYP2U, CYP4A, CYP4B, CYP4F, and CYP11B(209-212).

Early studies into CYP enzymes demonstrated they primarily transform EPA and DHA by epoxidation and hydroxylation(213,214). EPA is frequently metabolized into ω/(ω-1)-hydroxyeicosapentaenoic acids (HEPE) and epoxyeicosatetraenoic acids

(EEQs). DHA is often converted into ω(ω-1)-hydroxydocosahexaenoic acids (HDoHE) as well as EDPs. Furthermore, EPA and DHA compete with the n-6 PUFA arachidonic acid (AA) for CYP metabolism via binding sn-2 positions on glycerophospholipids across several different mammalian species including humans, rats, and mice(215,216). It has been demonstrated that nearly all CYP enzymes significantly involved with AA metabolism also act on EPA and DHA(216). A previous study showed that supplementing animals with dietary n-3 PUFAs resulted in altered membrane phospholipid composition(217). The human CYP isozymes that have the most well-characterized metabolic activity with respect to EPA and DHA include CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP2J2(218,219). When metabolising DHA, CYP2C8 produces a regioisomeric mixture of EDPs, with 19,20-EDP being the primary metabolite(217). 19,20-EDP is the not the dominant product of the other CYP2C isozymes, but they still exhibit significant epoxygenase activity(217,218). In contrast, CYP2J2 displays pronounced regioselectivity, primarily transforming DHA into 19,20-EDP(217,218). While the biological significance of this behaviour is yet to be elucidated, all of the aforementioned human CYP isoforms (except CYP2C8) stereo-selectively produce R,S-enantiomers when metabolizing n-3 PUFAs(219,220).

1.10 Bioactivity and -availability of EpFAs

The field of study surrounding the bioactivity of DHA metabolites is growing rapidly; Recent research has repeatedly demonstrated their cytoprotective properties(221-223). 19,20-EDP appears to exert particularly potent protection of cardiovascular health(221,224,225). However, lack of definitive mechanistic understanding regarding EDPs has hindered the clinical development of therapeutics based upon them. Previous

works indicate that EDPs activate BK channels in vascular smooth muscle cells (VSMCs) required for mesenteric and coronary microvessel dilation(226-229). In this model, EDPs were significantly more potent than epoxyeicosatrienoic acid (EET) compounds derived from AA(228). Other groups have also demonstrated that DHA-derived EpFAs could involve the activation of K_{ATP} channels(230-232). Some evidence indicates that EpFAs may sensitize TRPV1 and TRPA1 receptors via prostacyclin receptors(233). Another group has suggested that ethanolamide derivatives of 19,20-EDP evoke anti-inflammatory effects through the endocannabinoid receptor 2 coupled to beta-arrestin activity(234). Experimental data also indicates that EpFAs could influence gene transcription through direct interactions with peroxisome proliferator-activated receptors(235,236). Additionally, considerable work has been done to identify potential GPCRs that could be activated by fatty acids(237,238). Recent studies by our group indicate that sirtuins play an important role in mediating the cardioprotective effects of EpFAs(239-243). Notably, molecular targets of EpFAs may exhibit stereo- and/or regioselectivity with respect to the degree of activation that different metabolites may elicit(227). This is important to note given the tissue-specific expression of certain CYP isozymes that themselves stereo- and/or regioselectively produce EpFAs from their respective PUFA molecules.

Beyond a lack of established MoAs, the current therapeutic potential of EpFAs is limited by several issues regarding their bioavailability including autoxidation, β-oxidation, esterification, and metabolic conversion by soluble epoxide hydrolase (sEH) (244,245). EpFAs are frequently the target of inactivating β-oxidation and chain elongation reactions(246,247). The activity of EpFAs may also be limited by their significant binding

affinities for cytosolic fatty acid binding proteins(248). While sequestration within phospholipid membranes following esterification may theoretically hinder the bioactivity of EpFAs with respect to cytosolic, nuclear, and mitochondrial mediators, evidence seems to indicate that membrane-bound, phospholipase A2 (PLA2)-accessible CYP-eicosanoids are an important reserve pool of molecules available following ischemic injury(249-252). Encoded by the EPHX2 gene, sEH rapidly hydrolyzes EpFAs, converting them into vicinal diols which generally possess diminished bioactivity (244,245,253). Notably, recent studies are beginning to attribute cytotoxicity unto sEH-derived diol metabolites of EpFAs(254-258). Ubiquitously expressed within the cardiovascular system, sEH metabolizes virtually all CYP-derived EpFAs. Furthermore, upregulation of sEH stimulated by a plethora of risk factors appears to be a significant contributor to the pathogenesis of many CVDs (244,259-263). In the past twenty years, the cardioprotective benefits of genetic and pharmacological inhibition of sEH have become a massive point of interest for cardiovascular research(264-270). Additionally, CYP-derived EpFAs can also be subjected to secondary transformation by COX and LOX enzymes. While the potential effects of these derivatives are not well characterized or understood, the potential consequences of COX- and LOX-metabolism on EpFA bioactivity should be considered during investigation(271-274).

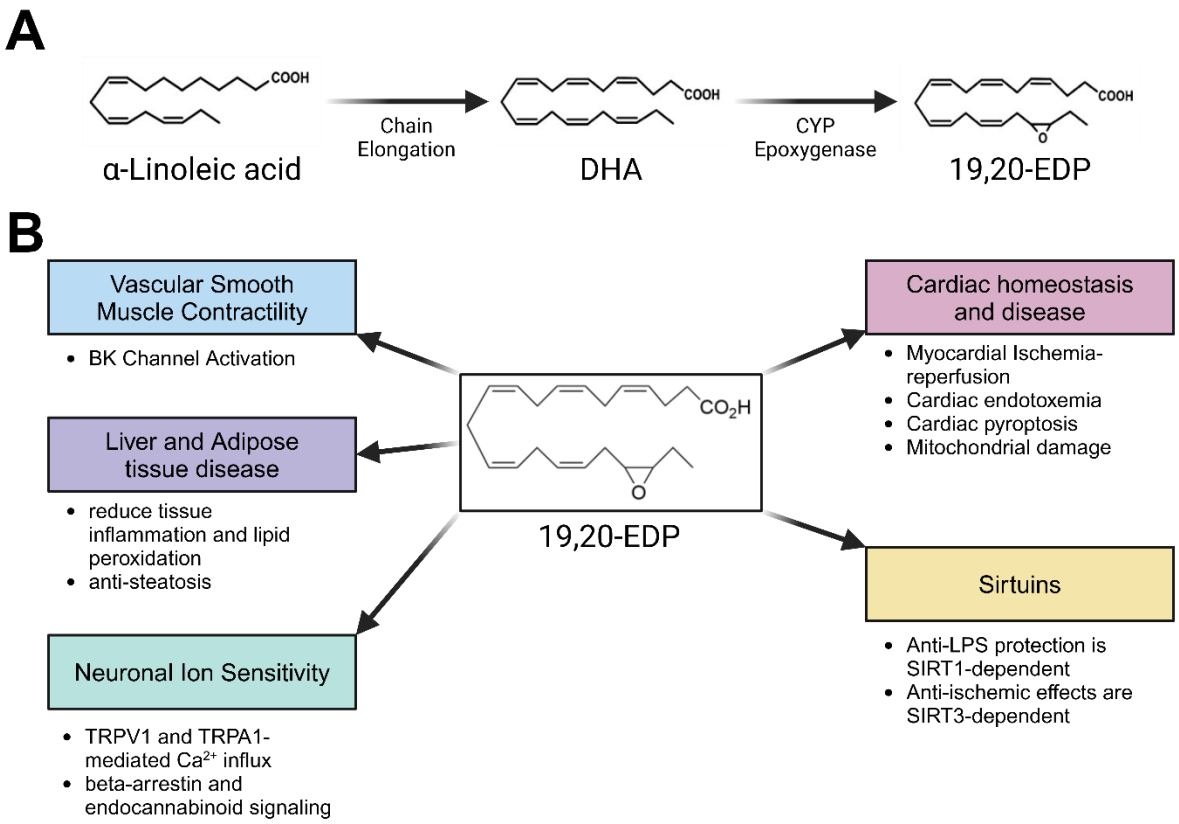


Figure. 1.3. *Biosynthesis and biological roles of 19,20-EDP.* (A) Biosynthetic pathway for 19,20-epoxydocosahexaenoic acid (19,20-EDP). α-Linoleic acid is an abundant n-3 polyunsaturated fatty acid (PUFA) derived from dietary sources. It is also frequently the target of chain elongation reactions, with one of the products being docosahexaenoic acid (DHA). DHA is then readily epoxidated by cytochrome P450 (CYP) epoxygenase enzymes, including CYP2J or CYP2C isoforms. The dominant product is 19,20-epoxydocosahexaenoic acid. (B) Biological roles of 19,20-EDP as discussed in Section 1.9 and using (275).

1.11 Epoxylipid-based therapeutic agents

Characterization of the obstacles facing the clinical utilization of EpFAs has led to the development of novel pharmacophores(173,276-278). Our group has previously demonstrated that synthetic analogs based upon the structure of 16,17-EDP were capable of exerting cardioprotective effects against IR injury dependent on SIRT3 activity(240). EET analogs seem to hold significant promise as anti-hypertensive therapeutic agents(279-284). Further study also showed that EET mimetic compounds could ameliorate various forms of renal injury(285-289). Additional experimental data from other groups indicate that EET analogs can limit infarct size following myocardial infarction(290,291). Increasing effort is going towards developing EpFA mimetics that possess sEH-inhibiting moieties within their structure(292-295). Indeed, we have also demonstrated that including a sEH-inhibiting urea group within the structure of 11,12-EET helped to potentiate its cardioprotective bioactivity(276). OMT-28, a first-in-class synthetic analog of 17,18-EEQ developed by OMEICOS Therapeutics GmbH, is currently undergoing a phase II clinical trial for potential indications against persistent atrial fibrillation(296). Prior to this, OMT-28 had also exhibited anti-septic and anti-arrhythmic properties(297,298). Recently we published experimental data indicating that OMT-28 is cardioprotective in several models including *in vitro* endotoxemia and HR injury, and *ex vivo* IR injury. Notably, the salutary effects of OMT-28 appear to be dependent on a plethora of mediators including PI3Kα, $G\alpha_i$, PPARα, and SIRT1(299).

To the best of our knowledge, no group has explored the therapeutic potential of synthetic 19,20-EDP analogs until this point. SA-22 is meant to serve as a first-generation, proof-of-concept compound to determine if the cardioprotective properties of native

19,20-EDP can be replicated with a synthetic analog. Thus, in lieu of including more pharmacologically potent structural modifications such as the urea moiety, we opted to simply saturate some of the carbon-carbon double bonds which naturally exist along the structural backbone of long chain PUFA species(300,301). While we did not test SA-22 for improved molecular stability and resistance to oxidation, this type of structural modification should theoretically make EpFAs less chemically labile, as the double bond between bisallylic carbons possesses a low activation energy requirement for hydrogen loss and free radical formation, making EpFAs naturally susceptible to autooxidation(301). Consumed by more than a third a of American adults, n-3 PUFA rich marine oils are the most popular dietary supplements in the United States. However, despite it being well characterized that oxidation of long chain fatty acids significantly reduces/alters their bioactivity, evidence has been presented suggesting that most commercially available marine oil supplements are often oxidized(302-304). As such, investigation into whether partial synthetic backbone saturation of EpFAs improves molecular stability and preserves their bioactivity is a highly relevant question that remains unanswered thus far.

1.12 Physiological relevance of sirtuins

Silent mating type information regulation 2 homologs (SIRTs) or ‘Sirtuins’ are a class of nicotinamide adenine dinucleotide (NAD^+)-dependent deacylase enzymes known to regulate a multitude of critical cellular processes, such as inflammation, mitobiogenesis, cell death, and gene transcription(305-307). Sirtuins can be more broadly characterized as class III histone deacetylases (HDACs), with seven distinct homologs of yeast Sir2 being expressed in mammalian cells (SIRT1-7) (308). While sirtuins localize to a wide range of cellular compartments, they all possess a highly conserved NAD^+ binding

domain(309). Thus, sirtuins can act as a metabolic switch, regulating cellular processes and metabolism across many locales(310).

SIRT1 is the closest mammalian homolog to yeast Sir2 and localizes to both the nucleus and cytoplasm. Targets of SIRT1 deacetylase activity include a plethora of stress response mediators such as peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), Foxo, p53, peroxisome proliferator-activated receptor gamma (PPARγ) LKB1, HIF-2α, and target of rapamycin complex-2 (TORC2) (311-313). Similarly localizing to the nucleus, SIRT2 plays a role in regulating chromosomal stability during mitosis(314,315). Characterized as a regulator of many cellular processes including inflammation, glucose homeostasis, and genome stability, SIRT6 typically associates directly with chromatin(316-318). Deficiency in SIRT6 is associated with advanced ageing and lifespan shortening(319). Last of the nuclear-localized sirtuins, SIRT7 primarily associates with condensed chromosomes and nucleoli. Much like SIRT6, deficiency in SIRT7 is marked by shortened lifespans. Interestingly, reduced SIRT7 expression appears to cause the development of cardiac hypertrophy and inflammatory cardiomyopathy in mice(320-322). In contrast to other sirtuins, SIRT3-5 are found primarily within mitochondria and can be regarded as mitochondrial stress sensors. Mitochondria sirtuins are known to heavily alter the activity of many metabolic enzymes(323). It is important to note that mouse SIRT3 exhibits partially divergent splicing and localization when compared to human SIRT3. Specifically, mice express a third splice variant of SIRT3 that lacks the mitochondrial localization signal which accumulates in the cytoplasm and to a lesser extent in the nucleus(324,325).

Notably, different to all other yeast Sir2 homologs, SIRT3 expression is correlated with elongated lifespan in humans. Furthermore, polymorphisms in the SIRT3 promoter are associated with different ageing outcomes(326,327). Given that CVD is the leading cause of death among adults older than 60, it is highly likely that SIRT3-mediated regulation of metabolic enzyme activity plays a role in preventing myocardial dysfunction. Due to high pH and presence of abundant acetyl-CoA, most mitochondrial proteins are acetylated under physiological conditions and as such are subject to SIRT3 activity(328,329). Subsequently, SIRT3 has been shown to be involved in many facets of mitochondrial biology, from dynamic fission/fusion and respiration to antioxidant processes and is critical for adaptive cellular responses to various stresses, such as ischemia(243,307,311,330). For instance, SIRT3 deacetylates and activates the antioxidant enzyme manganese superoxide dismutase ($MnSOD_2$) which converts superoxide into hydrogen peroxide(331-333). Furthermore, SIRT3 is known to increase the activity of family of forkhead transcription factor 3a (Foxo3a), resulting in increased expression of $MnSOD_2$ and catalase(334,335). Importantly, catalase along with glutathione peroxidase, facilitates the conversion of hydrogen peroxide into water(336).

Under normal physiological conditions, FAO is the preferred metabolic pathway within the heart(337). However, various CVDs (including IR injury) compromise the ability of the heart to properly oxidize long-chain fatty acids(338,339). To maintain cellular ATP levels, myocardial metabolism is increasingly augmented by glycolysis. As described earlier, the resulting acidification of cellular cytoplasm due to glycolysis confers susceptibility to reperfusion injury unto ischemic cardiomyocytes(340). Growing evidence suggests that SIRT3 is an important regulator of FAO under stress conditions(341,342).

Furthermore, a previous study observed a positive correlation between ATP synthesis and SIRT3 expression across several tissue- and stress-dependent contexts(343). While the exact mechanisms connecting SIRT3 to ATP synthesis are still being elucidated, it is commonly believed that SIRT3 directly improves enzyme activity by deacetylation. Beneficiaries of this activity include a multitude of enzymes central to mitochondrial respiration such as complex I (subunits NDFU9 and NDUFS1), long-chain acyl-CoA dehydrogenase (LCAD), and acetylcoenzyme A synthase 2 (AceCS2), complex II, glutamate dehydrogenase, and isocitrate dehydrogenase 2(341,343-346). In addition to its direct activity on metabolic enzymes, SIRT3 modulates cellular metabolism by regulating AMP-activated protein kinase (AMPK) activity(347-349). Upon phosphorylation by liver kinase B1 (LKB1), AMPK increases ATP production by upregulating cellular glucose import and FAO while simultaneously downregulating ATP-consuming anabolic pathways(350). AMPK is also known to activate PGC-1α, a well-known promoter of mitobiogenesis(351). Notably, both AMPK and LKB1 have been established as targets for deacetylation and activation by SIRT3(348,352). Interestingly, a previous study found that treatment with resveratrol (a well-characterized SIRT agonist) activated AMPK and prevented the development of cardiac hypertrophy *in vitro* and *in vivo*(353,354). Given the established relevance of SIRT3-mediated regulation of metabolism in several cardiac injury models, SIRT3 holds promise as a therapeutic target against ischemic injury (355-358).

Increasing interest is being directed towards the relationship between SIRT3 and apoptosis. While it is known that the primary mode of cell death within the infarct region following myocardial IR/HR injury is necrotic/pyroptotic in nature, apoptosis also

contributes to injury pathogenesis(359-361). Recent studies have begun to indicate that both nuclear full-length SIRT3 and mitochondrial short SIRT3 exert anti-apoptotic activity. The nuclear splice variant of SIRT3 commonly deacetylates histones H3-K9ac and H4-K16ac both in vitro and in vivo(362). However, the downstream consequences of this activity are still being elucidated. On the other hand, mitochondrial SIRT3 regulates the well-characterized lysine-acetylation-sensitize Ku70-Bax interaction that induces apoptosis. Briefly, acetylated Ku70 is unable to bind and sequester Bax, thus allowing it to translocate to the mitochondria where it causes the release of cytochrome c and subsequent activation of caspase-3 and -9(363). Interestingly, in contrast to other cell types and tissues, SIRT3 is the only SIRT isoform capable of deacetylating Ku70 in cardiomyocytes(324,364). Additionally, SIRT3 was demonstrated to deacetylate and downregulate activity of the pro-apoptotic protein p53 in vitro(365).

1.13 Sirtuin biochemistry

Perhaps the most important feature of all Sir2 homologs is their highly conserved 250 amino acid long Rossmann fold NAD^+-binding and catalytic core domain(310). This means that despite vastly divergent subcellular localization and expression, sirtuin-potentiating compounds should theoretically affect all sirtuins. The chemistry of sirtuins is somewhat unique within biology and has been the subject of study for the last two decades(310). The currently proposed mechanism of deacetylation by sirtuins is known as the ADPR-peptidyl-imidate mechanism(366). Starting from acetylated protein-bound sirtuin and NAD^+, the mechanism essentially describes a two-step process wherein the reactants first reversibly form an imidate intermediate product where the reaction can either decide to proceed forward with hydrolysis or reverse via attack of the intermediate

product by nicotinamide(366). In more detail, before a reaction can be initiated by NAD+ binding, sirtuins must be bound to an acetylated peptide. This is because in the absence of a bound peptide, the NAD^+ binding pocket cannot assume a catalytically active conformation(367,368). Upon peptide attachment, reactions begin with binding of NAD^+ to the catalytic core. Reactant preorganization is particularly important to this reaction as the binding of NAD^+ in an extended conformation positions its C1' carbon very close to the acetyl-lysine carbonyl oxygen, assisting reaction catalysis(369,370). From here, the imidate intermediate is formed by a one-step ADP-ribosylation of the acetyl-oxygen by NAD^+(371). Whether this reaction occurs via S_N2, S_N1, or highly asynchronous S_N2-type mechanisms remains a point of contention among biochemists(310). The formation of an ADPR-peptidyl-imidate intermediate forms a regulatory checkpoint for sirtuin-mediated deacetylation reactions(366). Specifically, deacetylation reactions can experience a form of auto-inhibition wherein upon oxidation of NAD^+, the NAM product immediately re-binds the catalytic pocket and attacks the C1' anomeric carbon, catalyzing a reverse reaction from imidate to NAD^+. The forward reaction is marked by nucleophilic attack by the imidate intermediate on the 2'-OH to resolve deacetylation(372). Possessing a weak base amide group with a pK_a below 0, the imidate has a good leaving group(373). However, NAM is a better base with a pK_a of 3 when thermodynamically competing for the ribosyl electrophile, and upon saturation of the enzyme, can force reactions backwards. Consequently, the Michaelis complex of the intermediate product is actually the favorable equilibrium(374). It is this aspect of sirtuin chemistry that describes how NAM acts as an inhibitor when added exogenously(375). This has been supported by previous studies utilizing isonicotinamide (isoNAM), a non-reactive isostere of NAM that can bind the same

catalytic site but cannot react with the imidate intermediate. These groups found that isoNAM enhanced sirtuin activity despite binding the inhibitory pocket and increased yeast replicative lifespan(376-378). Importantly, the physiological concentration of NAM in animals is sufficient to inhibit sirtuins *in vitro*(372,375,379).

The conserved NAM-binding C-pocket possesses two features which create the possibility of a direct activity-modulating interaction between sirtuins and PUFAs. First, the C-pocket is highly hydrophobic and would have high binding affinity for the long fatty acid carbon backbone. Second, several of the critical residues for NAM binding within the C-pocket are serines, which like to form hydrogen bonds with carboxylic acids, a structural commonality of all PUFAs(380,381). Interestingly, we have previously observed sirtuin dependency with respect to the cardioprotective bioactivity of 19,20-EDP *in vitro* and *ex vivo*(240,242).

1.14 Thesis overview

1.14.1 Rationale

Despite advances in reperfusion techniques and adjuvant therapies, mortality due to myocardial ischemia and reperfusion injury continues to be a significant clinical issue in globally. Positioned as terminally differentiated post-mitotic cells with exceptionally high metabolic demands, cardiomyocytes are particularly vulnerable to stressors that disrupt and damage mitochondria such as IR injury. As such, maintaining a healthy pool of cardiac mitochondria is vital to both ameliorating IR injury and lessening the broader clinical burden of myocardial ischemic attacks and subsequent chronic heart failure.

Dietary consumption of long chain n-3 PUFAs have long been correlated with positive outcomes regarding CVD in population studies. More recently, research groups

have demonstrated the cardioprotective properties of endogenously derived ‘epoxylipid’ PUFA metabolites. But despite extensive study, the structural, metabolic, and mechanistic heterogeneity amongst epoxylipids has confounded identification of their molecular targets and roles in cardiovascular diseases. Previously our group has demonstrated that the cardioprotective properties of 19,20-EDP were SIRT-dependent. Being that sirtuin enzymes are well-known for their protective effects with respect to mitochondrial health, EDPs are worth exploring in the context of myocardial ischemia. However, the rapid conversion of EDPs to less bioactive vicinal diols by sEH along with a plethora of other metabolic pathways such as β-oxidation and plasma membrane-esterification limits their current therapeutic utility. Thus, we have an interest in developing synthetic structural mimetics of 19,20-EDP that may possess improved bioavailability, their functional interactions with sirtuins, and the resulting impact on mitochondrial homeostasis in the context of hypoxic myocardial injury.

1.14.2 Hypothesis

We hypothesize that the structural 19,20-EDP analog SA-22 will protect cardiomyocytes against IR/HR injury by maintaining mitochondrial homeostasis and blunting innate immune responses dependent on the activity of SIRT3.

1.14.3 Objectives

We began this project with two primary objectives. First, we sought to replicate the cardioprotective effects of 19,20-EDP in murine *ex vivo* and *in vitro* models of IR/HR injury using a synthetic structural analog of 19,20-EDP SA-22. Second, we confirmed if the cardioprotective benefits of 19,20-EDP and its analog SA-22 were dependent on SIRT3 enzymatic activity.

CHAPTER 2. METHODS

2.1 Reagents

Stock solutions of 19,20-EDP, antimycin A, decylubiquinol, FCCP, nigericin, rotenone, and ubiquinone were prepared in absolute ethanol. Stock solutions of 3-TYP, digitonin, DUB, hoechst 33342, retinoic acid, SA-22, were prepared in DMSO. Reagents used for transfection (pCMV-dR8.2 dvpr, pMT-mKeima-Red, pCMV-VSV-G, pLJM1-eGFP, and LP2K), and laminin were prepared in D-PBS. Stock solutions of acetyl CoA, ADP, ascorbic acid, BSA, CHAPS, collagenase type II, cytochrome c, DCPIP, dextrose, DTT, DNase type I, DTNB, EDTA, glutamate, glycine, HEPES, malonic acid, nicotinamide, oxaloacetic acid, puromycin, pyruvate, sodium hydrosulfite, succinate, sucrose, tris acid, tris base, trypan blue, trypsin, and urea were prepared in sterile cell culture-grade water. Drugs were prepared such that vehicle concentrations did not exceed 0.1% v/v in media. A list of materials and reagents used is provided in **Table 1.**

Table 2.1. List of materials and reagents

Reagent	Supplier	Catalogue #
0.45um PES syringe filter	MilliporeSigma	SLHV033RS
10 cm plastic culture dishes	Sarstedt	83.3902
19(20)-EpDPA	Cayman Chemical	10175
20S Proteasome activity assay kit	MilliporeSigma	APT280
35mm glass-bottom dishes	MatTek	P35G-1.0-14-C
3-TYP	Selleck Chemical	58628
60mm culture dishes	Fisher Scientific	FB012921
6-well glass-bottom plates	MatTek	P06G-1.0-20-F
Acetylated Lysine antibody	Cell Signaling Technology	cs9441
Acetyl CoA lithium salt	Sigma Aldrich	A2181
ADP sodium salt	Sigma Aldrich	A2754
α-Tubulin antibody	Abcam	ab4074

Anti-mouse HRP-conjugated antibody	Cell Signaling Technology	7076
Anti-rabbit HRP-conjugated antibody	Cell Signaling Technology	7074
Antimycin A	Sigma Aldrich	A8674
Ascorbic acid	Sigma Aldrich	A5960
Beta-actin antibody	Cell Signaling Technology	4967
Bleach	Lavo	02358107
BLUelf prestained protein ladder	FroggaBio	PM008-0500F
BME	BioShop	MER-002
Bradford protein dye concentrate	Bio-Rad	5000006
BSA	Sigma Aldrich	A6003
BSA fatty acid free	Sigma Aldrich	A7030
Calcium chloride	Caledon Laboratories	2521-1
cTnT antibody	Cell Signaling Technology	cs5593s
Caspase-1 fluorogenic substrate (Ac-YVAD-AMC)	Enzo Life Sciences	ALX-260-024-M005
Cell scrapers	Thermo Fisher Scientific	179693
Cell strainer (70um Nylon Mesh)	Fisher Scientific	22363548
CHAPS	Sigma Aldrich	226947
Collagenase Type II	Worthington Chemical Corporation	LS004176
CoralHue® mitochondria-targeted mKeima-Red (pMT-mKeima-Red)	MBL Life Science	AM-V0251M
COX IV antibody	Cell Signaling Technology	cs11967s
CS antibody	Abcam	ab129095
Cytochrome c (cytochrome c from equine heart)	Sigma Aldrich	C2506
DCPIP	Sigma Aldrich	D1878
Dextrose [anhydrous]	EMD	D-4751
Digitonin	Sigma Aldrich	D141
DMSO	Sigma Aldrich	D2650
DRP-1 antibody	Santa Cruz	sc-271583
DTT	Sigma Aldrich	646563
DMEM (Dulbecco's Modified Eagle Medium) [high glucose]	Gibco	11965-092
DMEM (Dulbecco's Modified Eagle Medium) [low glucose]	Gibco	11885-084
DMEM/F12	Gibco	11320-033

DNase I	Worthington Chemical Corporation	LS002139
D-PBS	Thermo Fisher Scientific	14190144
DTNB	Research Organics	2180D
DUB	Sigma Aldrich	D79113
Ethanol (95%)	Commercial Alcohols	P016BA95
EDTA dihydrate	Sigma Aldrich	ED2SS
Euthanyl© Sodium pentobarbital injection	Bimeda-MTC Animal Health	00141704
FCCP	Abcam	ab147482
FBS	Thermo Fisher Scientific	12483-020
GSDMD antibody	Santa Cruz	sc-393581
Glutamate (L-Glutamic acid potassium salt monohydrate)	Sigma Aldrich	G1501
Glycerol	Caledon Laboratories	5350-1
Glycine	Fisher Scientific	BP3815
H9c2 cells	ATCC	CRL-1446
HDAC fluorogenic substrate	BPS Bioscience	50032
HEK293T cells	ATCC	CRL-3216
HEPES	Fisher Scientific	BP310
Hoechst 33342	Thermo Fisher Scientific	62249
Horse serum (New Zealand)	Gibco	16050-122
HSP60 antibody	Cell Signaling Technology	cs4870
Immersol™ Immersion Oil 518F	Carl Zeiss AG	444960
Immun-Blot® PVDF Membrane	Bio-Rad	1620177
Instant skim milk powder	Smucker Foods	3808
IL-1β antibody	Abcam	ab9722
Laemmli sample buffer	Bio-Rad	1610747
Laminin	Sigma Aldrich	L2020
LC3B antibody	Cell Signaling Technology	cs3868
LP2K	Thermo Fisher Scientific	11668019
Magnesium sulfate [anhydrous]	Fisher Scientific	M65-500
Malate (L-(-)-Malic acid)	Sigma Aldrich	M1000
Malonic acid	Sigma Aldrich	M1296
Methanol	Sigma Aldrich	34860
Mini-PROTEAN TGX™ Gels (12%)	Bio-Rad	4561094
Mini-PROTEAN TGX™ Gels (4-20%)	Bio-Rad	4561094

MiR05	Oroboros Instruments	60101-01
$MnSOD_2$ antibody	Abcam	ab13533
$MnSOD_2$ antibody (acetyl)	Abcam	ab137037
NAM	Sigma Aldrich	72340
OPA1 antibody	BD Biosciences	bd612606
Opti-Mem reduced serum media	Thermo Fisher Scientific	31985070
Oxaloacetic acid	Calbiochem	5000
P62 antibody	Abcam	ab5646
Parkin antibody	Cell Signaling Technology	cs2132
pCMV-dR8.2 dvpr plasmid	Addgene	8455
pCMV-VSV-G plasmid	Addgene	8454
Penicillin-Streptomycin (10000U)	Thermo Fisher Scientific	15140122
Pierce™ protease and phosphatase inhbibitor mini tablets	Thermo Fisher Scientific	A32959
PINK1 antibody	Abcam	ab23707
pLJM1-eGFP plasmid	Addgene	19319
Potassium chloride	Fisher Scientific	P-217B
Potassium cyanide	Sigma Aldrich	60180
Potassium ferricyanide	Electron Microscopy Sciences	20150
Potassium phosphate monobasic	Caledon Laboratories	6660-1
Puromycin	Sigma Aldrich	P8833
Retinoic acid	Sigma Aldrich	R2625
Rotenone	Sigma Aldrich	R8875
SA-22	Falck Lab	N/A
SIRT1 antibody	Cell Signaling Technology	cs9475s
SIRT3 antibody	Cell Signaling Technology	cs5490s
SIRT3 human recombinant enzyme	BPS Bioscience	50014
SIRT3 fluorogenic assay kit	BPS Bioscience	50088
SIRT assay buffer	BPS Bioscience	50090
SIRT developer solution	BPS Bioscience	50032
Sodium azide	Sigma Aldrich	S-2002
Sodium bicarbonate	Sigma Aldrich	712519
Sodium chloride	Sigma Aldrich	746398
Sodium dithionite	Sigma Aldrich	71699
SDS	Fisher Scientific	BP8200
Sodium fluoride	Sigma Aldrich	S7920

Sodium hydrosulfite	Sigma Aldrich	157953
Sodium phosphate dibasic	Caledon Laboratories	8120-1
Sodium phosphate monobasic	Caledon Laboratories	8180-1
Sodium pyrophosphate	Sigma Aldrich	P8010
Sodium pyruvate	Sigma Aldrich	P5280
Stericup® Quick Release 0.22um Filters	MilliporeSigma	S2GPU5RE
Succinate (dibasic sodium salt)	Sigma Aldrich	14160
SDH-A antibody	Cell Signaling Technology	cs5839s
Sucrose	Caledon Laboratories	8720-1
SuperSignal™ West Pico PLUS chemiluminescent substrate	Thermo Fisher Scientific	34580
T25 cell culture flasks	Sarstedt	83.3910.002
T75 cell culture flasks	Sarstedt	83.2911.002
Tris acid	Fisher Scientific	BP153-500
Tris base	Invitrogen	15504-020
Trypan Blue (0.4%)	Thermo Fisher Scientific	15250061
TrypLE™ Express Enzyme, 1X	Gibco	12605028
Trypsin	Worthington Chemical Corporation	LS003707
Tween-20	Sigma Aldrich	P7949
Ubiquinone	Sigma Aldrich	C7956
Urea	Sigma Aldrich	0000279909
VDAC antibody	Abcam	ab14734
Zeiss Lens Cleaner	Carl Zeiss Vision	740.000.0014 6

2.2 Synthesis of the 19,20-EDP analog SA-22

(10*Z*,16*Z*)-18-(3-Ethyloxiran-2-yl)octadeca-10,16-dienoic acid (SA-22) was synthesized by the Falck lab at ≥95% purity using established synthetic methods and characterized using ^{1}H/ ^{13}C Nuclear Magnetic Resonance (NMR) and mass spectroscopy(293,297,382,383). The synthetic process for SA-22 can be found among the supplementary digital information published alongside Kranrod et al.(239).

2.3 Animals

All experiments used young, male wildtype (WT) C57BL/6J mice weighing 25-35g, aged between 2-6 months. The mouse colony was housed and maintained at the University of Alberta under stable conditions with regards to temperature and humidity on a 12:12-h light-dark cycle. Mice were given standard rodent chow ad libitum (fat 11.3%, fiber 4.6%, protein 21% (w/w)). Dietary composition included linolenic acid (0.27%), linoleic acid (2.12%), arachidonic acid (0.01%), omega-3 fatty acid (0.45%), total SFA (0.78%), and total MSFA (0.96%) (PicoLab®Rodent Diet 20 Cat. No 5053, LabDiets, Inc., St. Louis, MO, USA). All animal experimental protocols were approved by the University of Alberta Health Sciences Welfare Committee (University of Alberta Animal Welfare, ACUC, study ID#AUP330) and conducted according to strict guidelines provided by the Guide to the Care and Use of Experimental Animals (Volume. 1, 2nd ed., 1993, from the Canadian Council on Animal Care).

2.4 Isolated heart perfusions

Mice were anesthetized via intraperitoneal injection with sodium pentobarbital (Euthanyl™, 100mg/kg). Mice were then monitored for complete non-responsiveness to external stimulation before the hearts were excised and perfused in Langendorff mode with Krebs-Henseleit buffer (pH 7.4) (in mM) 120 NaCl, 25 $NaHCO_3$, 10 Dextrose, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 4.7 KCL, 2 Sodium Pyruvate and bubbled with 95% O_2, and 5% CO_2 at 37°C(221,384). Following hanging of the heart, the left atrium was excised, and a water-filled, plastic saran-wrap balloon was inserted into the left ventricle via the mitral valve. Connection of the balloon to a pressure transducer allowed for continuous monitoring of LVDP (cm H_2O) and heart rate (HR) (beats/min). Hearts were omitted from

experimental analysis if they failed to sustain an LVDP greater than 80 cm H_2O or had a persistent arrhythmia. Perfusions were done in retrograde at a constant flow rate for 20 minutes at baseline before 30 minutes of global no flow ischemia followed by 40 minutes of reperfusion. Starting at reperfusion hearts were perfused with vehicle (final concentration less than 0.1% DMSO), SA-22 (1 μM), or 19,20-EDP (1 μM). Some hearts were perfused with SA-22 (1 μM) together with either the pan-sirtuin inhibitor nicotinamide (NAM, 30 μM) or the SIRT3-selective inhibitor 3-(1H-1,2,3-triazol-4-yl) pyridine (3-TYP, 50 μM). Previous studies with CYP-derived *n*-3 EpFAs demonstrating cardioprotective benefits informed our choice of concentrations(385,386). Chemicals were added to the apparatus 10 minutes before ischemia and were present in the heart throughout the reperfusion period. Tissues were immediately snap frozen in liquid nitrogen for tissue analysis following reperfusion. Postischemic functional recovery was assessed by taking the percentage of left ventricular developed pressure (LVDP) at 40 minutes into reperfusion as a percentage of baseline LVDP. Software from ADI (AD Instruments, 4360 Arrowswest Drive, Colorado Springs, Colorado) was used to acquire and analyze haemodynamic parameters. Isolated heart perfusion experiments were graciously performed by Dr. Ahmed Darwesh, Dr. Wesam Bassiouni, and Andy Huang.

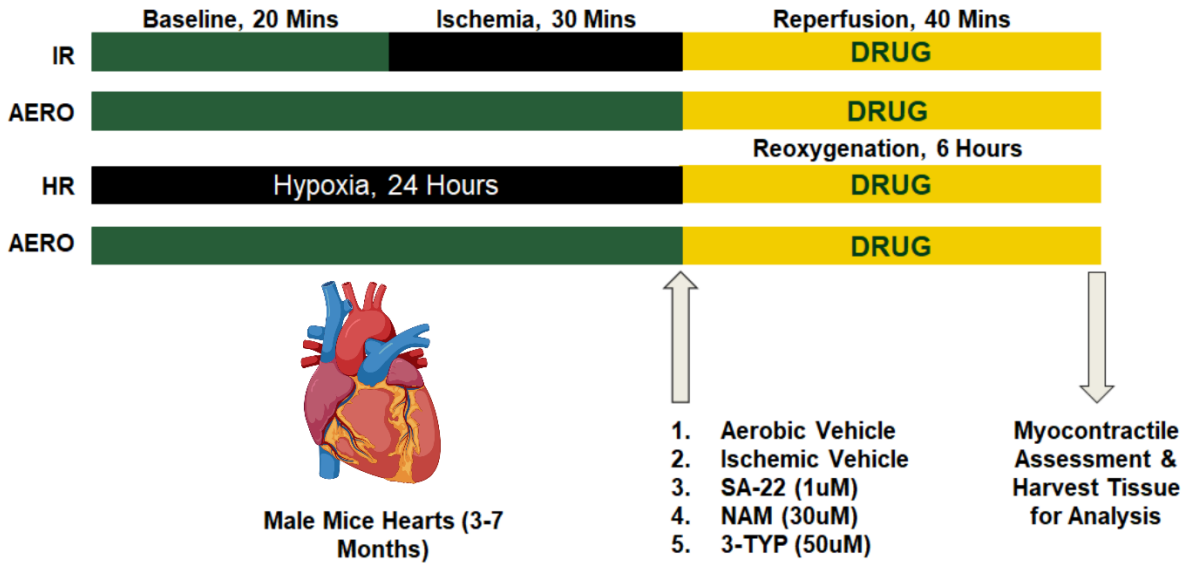


Figure 2.1. Ischemia-reperfusion and hypoxia-reoxygenation experiment design.

2.5 Cultivation of H9c2 cardiac cells and treatment protocols

H9c2 rat embryonic myoblast cells (Sex Unspecified) were purchased from the American Type Culture Collection (ATCC) and maintained between 30 to 90% confluency in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell differentiation was induced by supplementation of media with 10 nM all-trans retinoic acid for 14 days. Differentiation of cells was confirmed by densitometric blot analysis of cardiac Troponin T, comparing cells that did or did not receive retinoic acid(387). Cells were plated at a density of 1.0×10^6 cells/mL and culture conditions were maintained at 37°C, 5% CO_2 and 95% air. Cell viability was assessed with a trypan blue exclusion test. H9c2 cells subjected to hypoxia-reoxygenation (HR) or normoxia were treated with the following agents: 19,20-EDP (1 μM), SA-22 (1 μM) and/or either NAM (30 μM) or 3-TYP (50 μM).

2.6 Trypan blue exclusion assay

H9c2 cardiomyblasts in 10cm dishes (8ml culture volume) were treated as described in Section 2.5 prior to harvesting. Following dissociation of cells from their culture flasks and neutralization with fresh DMEM, 100µl of each cell suspension was mixed with 100µl of Trypan blue solution. Trypan blue exclusion assays were then performed using a hemocytometer. Viable cells were trypan blue-negative while non-viable cells were trypan-blue positive. Percent cell viability was calculated as (viable cells / total cells).

2.7 Lentivirus transfection and stable Mito-Keima expression in H9c2 cells

To construct the pLJM1-mito-Keima transfer plasmid, the mito-Keima open reading frame from the CoralHue® Mitochondria-targeted mKeima-Red plasmid was subcloned into an empty pLJM1 vector backbone. Transfection complexes were formed by incubating 15000 ng pLJM1-mito-Keima plasmid, 6000 ng pCMV-dR8.2 dvpr packaging plasmid, 3000 ng pCMV-VSV-G envelope plasmid, and Lipofectamine 2000 Transfection Reagent in 750µl Opti-MEM at room temperature for 30 minutes. Transfection complexes were then incubated with wild-type HEK293T cells in T75 flasks with 10 ml DMEM supplemented with 10% FBS and 100U/ml penicillin/streptomycin for 24 hours at 37°C and 5% CO_2. Lentivirus production was facilitated by replacing cell culture media the following day. Lentivirus-containing DMEM was collected after another 24 hours. Media was cleared of HEK293T cells by centrifugation at 500 x *g* for 5 minutes followed by filtration through a 0.45 µm PES syringe filter. H9c2 cells were seeded in 6-well plastic culture plates and grown to 60% confluency whereupon the culture media was replaced with 2 ml of lentivirus-containing DMEM. Cells were spinfected by centrifuging

6-well plates at 1000 x *g* for 45 minutes at 37°C. Lentivirus-containing media was aspirated after 24 hours and replaced with fresh DMEM. After another 24-hour period, successfully transfected cells were isolated by supplementing media with 2 µg/ml puromycin. Once cells reached 80-90% confluency they were seeded into larger dishes for further cultivation and frozen in liquid nitrogen for long-term cryostorage. Stable mito-Keima expression was validated using fluorescence microscopy.

2.8 Isolation of neonatal rat cardiomyocytes (NRCM)

Primary cultures of cardiomyocytes were prepared from 2–3-day-old neonatal Sprague-Dawley rats (mixed sex) as previously described(388). Briefly, hearts were excised immediately following decapitation and placed in ice-cold D-PBS. After thorough rinsing, atria, fats, and connective tissue were discarded. The remaining ventricles were minced using scissors and then placed into a T25 tissue culture flask containing ice-cold D-PBS, DNase (0.025% w/v), collagenase (0.10% w/v), and trypsin (0.05% w/v). Tissue suspensions were digested on a rotary shaker at 37°C for 25 minutes and then transferred to a 50-mL Falcon™ tube containing 20 mL of DMEM/F12 media supplemented with 20% FBS. The tissues were centrifuged at 200 x *g* for 4 minutes at 4°C. After discarding the supernatant, the digestions were repeated two more times but with the supernatant being collected after the second and third digestions. Following the final digestion, tissue and supernatant fractions were combined and centrifuged at 1700RPM for 7 minutes at 4°C. The resulting pellet was resuspended in 20ml of DMEM/F12 media supplemented with 10% horse serum, 5% FBS, and 100U/ml of penicillin/streptomycin and passed through a 70 µm nylon mesh strainer. NRCMs were then seeded and cultivated on dishes coated

with laminin (10μg/ml). Cultures were used for experiments 48-72 hours after the initial plating.

2.9 Hypoxia-reoxygenation exposure

H9c2 cells and NRCMs were placed in a computer-controlled humidified hypoxic chamber (0.9% O2, 5% CO_2, and 94% N_2) for 24 h followed by reoxygenation under normoxic conditions (21% O_2/5% CO_2) for 6 h as described previously(243). Control cells were exposed to 30 h of normoxia. All hypoxic treatments used deoxygenated medium. The hypoxia chamber and controller were custom-designed and assembled in the instrumentation workshop at the Faculty of Pharmacy, University of Alberta, Edmonton, AB, Canada.

2.10 Immunoblotting

Subcellular fractions were isolated from frozen heart tissues and dissociated cell cultures. Mouse hearts were ground with a mortar and pestle on dry ice and homogenized in an ice-cold homogenization buffer (20 mM Tris-HCl, 50 mM NaCl, 50 mmol/L NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 250 mmol/L sucrose plus Pierce™ Protease and Phosphatase Inhibitor Mini Tablet (PPI), pH 7.0) as previously described(264). H9c2 cells and NRCMs were lysed using standard lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM $NaHPO_4$, 10 mM Tris-HCl, 250 mM sucrose, and PPI) as previously described(389). Cellular debris was separated via centrifugation at 800 x *g* and 4°C for 10 minutes. Plasma membrane associated proteins such as Gasdermin D contained in the debris pellet were re-solubilized with homogenization buffer containing 8M urea. Subsequent centrifugation of the supernatant at 10,000 x *g* for 20 minutes produced mitochondrial-

enriched pellets. Mitochondrial pellets were resuspended in homogenization buffer for analysis. Ultracentrifugation of the supernatant at 105,000 x *g* for 60 minutes separated microsomal membranes from the cytosol. Total protein concentration was assessed via Bradford assay, and Western blotting was carried out as previously described(221). Sample proteins (15-50ug) were reconstituted in Laemmli sample buffer and then boiled for 5 minutes. Samples were then loaded onto Mini-PROTEAN TGX™ gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were resolved by SDS-PAGE (90V, 2-3 hours) within an ice-bath prior to electrophoretic transfer (25V, overnight) onto Immun-Blot™ polyvinylidene difluoride (PVDF) membranes. Prior to probing, membranes were blocked with tris-buffered saline tween solution (TBST; 50 mM Tris-HCl (pH = 7.6), 150 mM NaCl, 0.1% Tween-20) with 5% BSA. Following blocking, membranes were incubated overnight with primary antibodies against SDHA-1 (1/2000), COX IV (1/5000), Citrate Synthase (1/5000), SIRT3 (1/1000), SIRT1 (1/1000) total $MnSOD_2$ (1/1000), acetyl-$MnSOD_2$ (1/1000), acetyl-Lysine (1/1000), OPA1 (1/1000), IL-1β (1/500), GSDMD (1/500), NLRP3 (1/500), VDAC (1/2000), β-Actin (1/2000), α-Tubulin (1/2000), cardiac Troponin T (1/500), DRP-1 (1/1000), PINK1 (1/1000), Parkin (1/1000), P62 (1/1000), and LC3B (1/1000). After washing, membranes were incubated with corresponding secondary antibodies made in TBST + 5% instant skim milk powder. Primary and secondary antibodies used are included in **Table 1**. Blots were visualized using the chemiluminescent substrate SuperSignal™ West Pico PLUS and a ChemiDoc MP Imaging System. Densitometric analyses were performed using ImageJ (NIH, USA). Membranes were re-probed following incubation with stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20, pH = 2.2) and 1 hour re-blocking.

2.11 Enzymatic assays

2.11.1 Spectrophotometric ETC complex assays

Spectrophotometry was employed to assess mitochondrial respiratory chain (RC) enzymatic function as previously described(390). Mitochondrial heart samples were assessed for complex I, II, III, IV, and CS activities. Complex activity was normalized to volume and protein concentration.

2.11.2 Sirtuin activity

Mitochondrial SIRT3 and cytosolic SIRT1 activity was detected according to the manufacturer's instructions using a fluorescent assay kit (50088 and 50081 respectively, BPS Bioscience, San Diego, CA, USA). This assay involves mixing samples with a specific fluorogenic HDAC substrate, bovine serum albumin, NAD^+, and proprietary assay buffer. Upon initial deacetylation by SIRT and subsequent exposure to the assay developer solution, the HDAC substrate produces a fluorescent product that can be measured using a fluorometric plate reader at 350-380/440-460 nm excitation/emission wavelengths. Replacing biological samples with the recombinant SIRT3 enzyme supplied the kit enabled us to examine the direct effect of SA-22 and 19,20-EDP on SIRT3 catalytic activity. SIRT activity was expressed as a fold of the aerobic vehicle group in relative luminescence units (RLU).

2.11.3 Caspase-1 activity

Cytosolic Caspase-1 activity was assessed as a marker of NLRP3 inflammasome complex activation. Activity in heart tissue lysate is proportional to the generation of a

fluorescent signal following cleavage and release of 7-amino-4-methylcoumarin (AMC) from a tagged caspase-1 peptide substrate, Ac-YVAD-AMC over time. Briefly, cytosolic samples (20-30 µg protein) were incubated with the fluorogenic substrate in a reaction buffer (50 mM HEPES, 100 mM NaCl, 0.5% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT) and the fluorescence intensity of the cleaved AMC was quantitated using a fluorometer (excitation 380 nm, emission 460 nm wavelengths). Activity was normalized to protein concentration then expressed as a fold of the aerobic vehicle treatment group in relative luminescence units (RLU). This protocol has been described previously(391).

2.11.4 20S proteasome activity

Total 20S proteasome activity assay was determined in cytosolic fractions monitoring the release of AMC by proteolytic cleavage of the peptide Suc-LLVY-AMC (APT280, Chemicon) by 20S proteasomes. The kit measures the formation of 7-Amino-4-methylcoumarin (AMC) from cleavage of substrate LLVY-AMC by the proteasome. Fluorescence was monitored at wavelengths of 380 nm (excitation) and 460 nm (emission). Activity was normalized to protein concentration then expressed as a fold of the aerobic vehicle treatment group in RLU. This protocol has been employed previously(389).

2.12 Microscopy

2.12.1 Live-cell fluorescence microscopy

For fluorescent microscopy experiments, H9c2 cells were plated on 6-well glass-bottom plates. Experimental treatments were conducted as described in **Sections 2.5** and **2.7**. For the last 30 minutes of hypoxia/normoxia, cells were incubated with 1 µM

Hoechst 33342 dye to illuminate nuclear DNA. Due to difficulties in maintaining cell-cultures on glass-bottom dishes for several weeks, all microscopy experiments utilized non-differentiated H9c2 cells. In all experiments, cells were imaged using a Zeiss Axio Observer Z1 inverted epifluorescence microscope fitted with a microincubator (37°C, 5% CO_2), using a 40X/1.3NA oil DIC objective lens and the Colibri 62 HE BFP GFP McRed filter cube. Images were captured using the Zeiss Zen© 2.3 (blue edition 2011) software package (Version 2.3.69.1018).

2.12.2 Quantification of Mito-Keima fluorescence

Fluorescence images were generated according to the following parameters: Neutral pH mitochondria fluorescence was excited at 470nm (25% intensity, 750ms exposure) and captured at 604nm. Acidic pH mitochondrial fluorescence was excited at 590nm (80% intensity, 800ms exposure) and captured at 604nm. Hoechst 33342 (DNA) fluorescence was excited at 365nm (10%, 100ms exposure) and captured at 455nm.

Before image export, fluorescence histograms were adjusted to isolate signal attributable to mitophagosomes from background noise. Analysts adjusting the images were blinded to their treatments. CZI images were then split into individual channels and exported as high-resolution TIFF files for analysis using ImageJ. Acidic channel images were then converted into a binary mask and discrete objects were counted. Acidic object counts were normalized to nuclei counts manually obtained from Hoechst 33342 channel images.

2.13 High-resolution respirometry

Mitochondrial respiratory function was assessed in H9c2 cells using an Oroboros-O2k high-resolution respirometer (OROBOROS Instruments, Innsbruck, Austria) according to the substrate-uncoupler-inhibitor titration (SUIT) protocol 008(151). Following experimental treatment protocols, cells were washed twice with PBS then dissociated with TrypLE™. After neutralizing TrypLE™ with fresh DMEM and collecting cell suspensions, cell density was calculated using the trypan-blue exclusion assay described in **Section 2.6**. Remaining cells were centrifuged at 500 x *g* for 4 minutes. Pellets were resuspended in mitochondrial respiration medium (MiR05, pH = 7.1, 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 1 g/L fatty acid-free BSA, dissolved in double-distilled water (ddH_2O)). 2.0 ml of cell suspension was then added to closed chambers at 37°C and 750RPM stirrer speed. Cells were given 20 minutes to equilibrate within the chambers before the basal respiratory state was recorded. Selective permeabilization of cytosolic membranes was achieved by incubating cells with 4 µM digitonin for 20 minutes prior to commencement of the SUIT protocol. Oxygen consumption rates (OCRs) at each respiratory state were recorded in the following order: (i) LEAK respiration stimulated by 5 mM pyruvate and 2 mM malate [PM_L]; (ii) OXPHOS stimulated by 2.5 mM adenosine diphosphate (ADP) [PM_P]; (iii) Mitochondrial outer membrane integrity was tested by addition of 10 µM cytochrome c [PMc_P]; (iv) Complex I respiration was saturated with 10 mM glutamate [PMG_P]; (v) Maximal complex I+II OXPHOS respiration following 10 mM succinate addition [$PMGS_P$]; (vi) Maximal non-coupled respiration (ET) following several step-wise 0.1 µM FCCP titrations [$PMGS_E$]; (vii) Non-coupled complex II respiration with

0.5 μM rotenone [S_E]; (viii) Residual oxygen consumption (ROX) after 2.5 μM antimycin A titration(392). Reported OCRs were calculated by normalizing all O_2 flux values to ROX respiration and cell count ([pmol O_2] / [sec*million cells]).

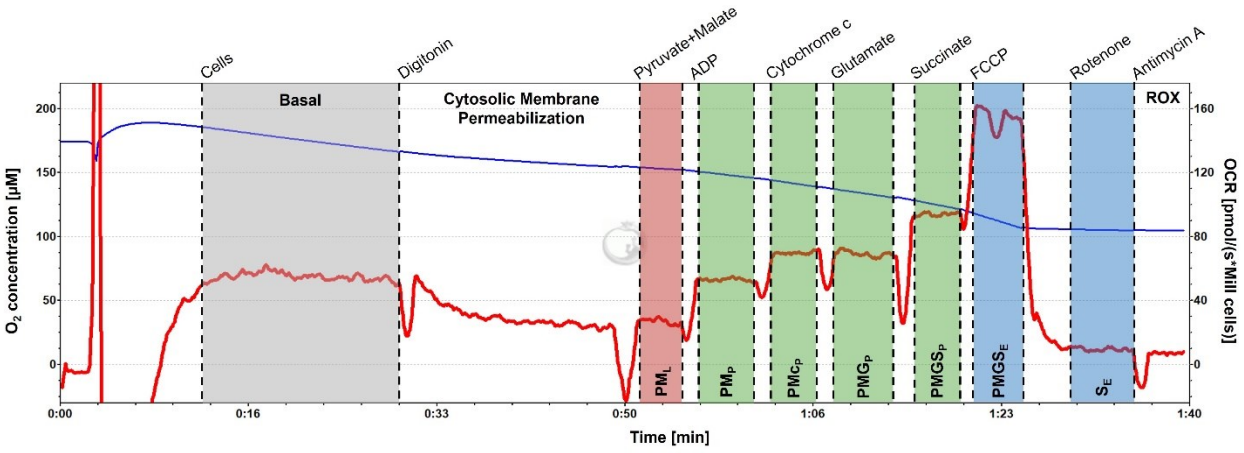


Figure 2.2. *Representative high-resolution respirometry experiment.* Oxygen consumption rates (OCRs) are measured at different respiratory states induced by various substrates, uncouplers, and inhibitor titrations.

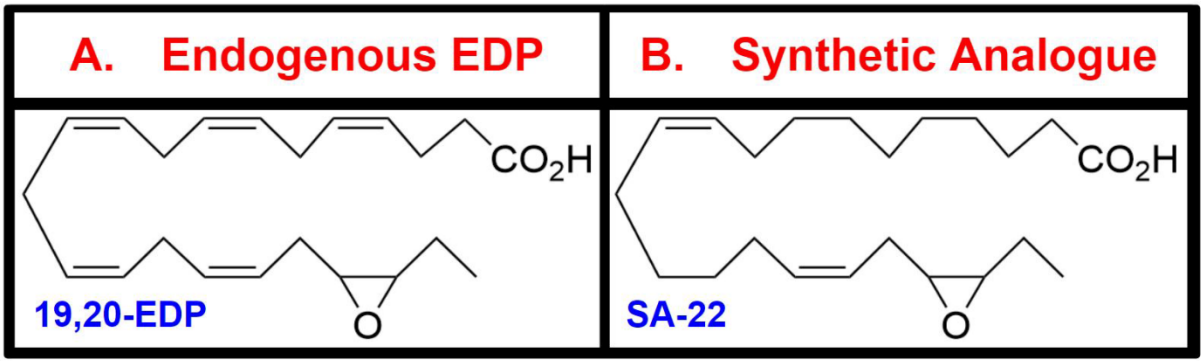
2.14 Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (Version 10.2.0(392)). Values are presented as mean ± standard error of mean (SEM). Statistical significance was determined by one-way ANOVA with a Bonferroni post-hoc test when comparing two or more groups to a control mean; $p < 0.05$ was considered statistically significant. Individual points on data plots/graphs represent separate biological replicates.

CHAPTER 3. RESULTS

3.1 19,20-EDP and SA-22 directly potentiate SIRT3 catalytic activity in vitro

In addition to growing evidence that the cardioprotective effects of 19,20-EDP were SIRT-dependent(240,243), unpublished preliminary work by our group suggested that both 19,20-EDP and SA-22 could directly bind to SIRT3 and modulate its activity. Thus, we employed the SIRT3 fluorogenic activity assay described in Section 2.11.2, incubating recombinant human SIRT3 (hSIRT3) enzyme in the presence of either 19,20-EDP or SA-22. Interestingly, both 19,20-EDP and SA-22 improved the catalytic capacity of hSIRT3, but only at 100 μM rather than the 1 μM concentration at which we have previously observed beneficial outcomes from EpFA treatment. Furthermore, potentiation of SIRT3 activity was completely abrogated by 3-TYP addition **(Figure 3.1C)**.


A. Endogenous EDP
B. Synthetic Analogue
CO2H
19,20-EDP
O
CO2H
SA-22
O

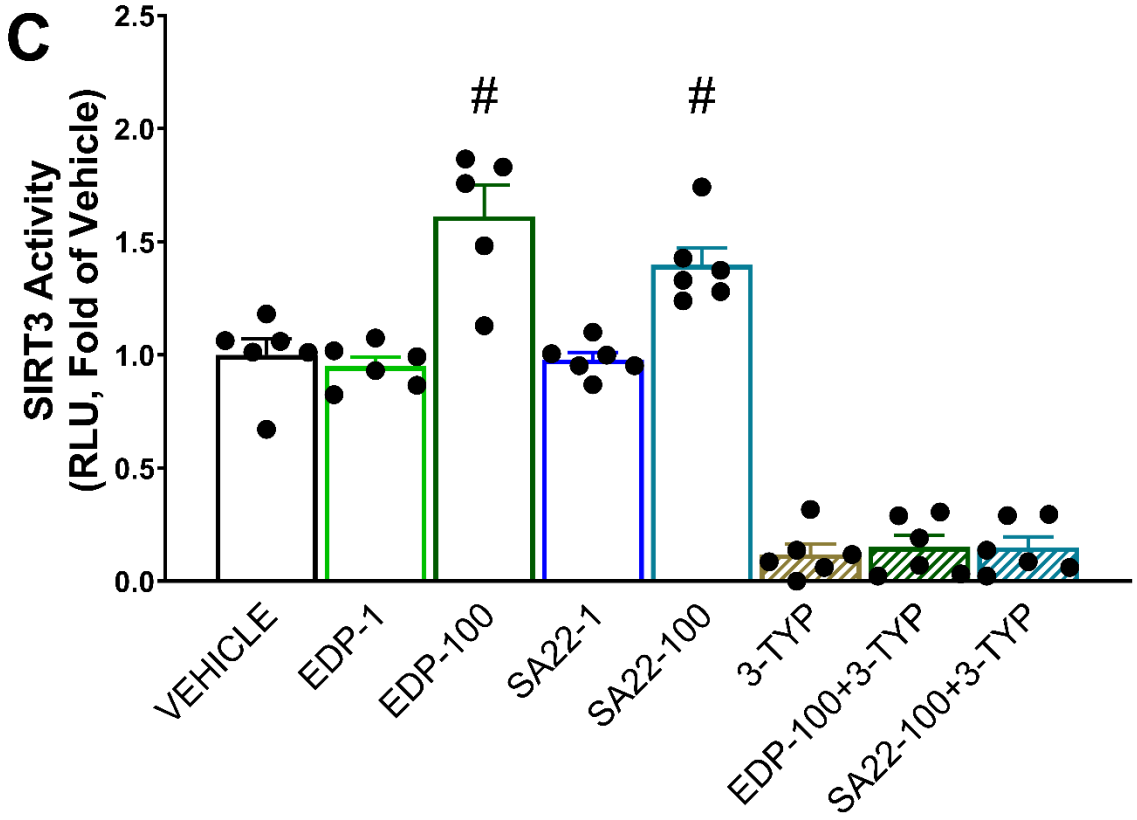

C
SIRT3 Activity
(RLU, Fold of Vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
#
#
VEHICLE
EDP-1
EDP-100
SA22-1
SA22-100
3-TYP
EDP-100+3-TYP
SA22-100+3-TYP

Figure 3.1. *SA-22 is a structural 19,20-EDP mimetic that boosts SIRT3 activity.* Chemical structures of an endogenously abundant metabolite of DHA, 19,20-EDP (A) as well as the metabolically more stable, synthetic analog SA-22 (B). (C) Human recombinant SIRT3 activity was assessed after incubation with either 19,20-EDP (1, 100 μM) or SA-22 (1, 100 μM), in the presence or absence of 3-TYP (50 μM) *in vitro*. Values represent mean ± SEM; # *p<0.05* vs. VEHICLE (n = 5-6 per group). 3-TYP; 3-(1H-1,2,3-triazol-4-yl) pyridine, DHA; Docosahexaenoic acid, EDP; Epoxydocosapentaenoic acid, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid.

3.2 Post-ischemic functional recovery is improved by SA-22 treatment

To prove the functional improvement in SIRT3 activity stimulated by the EDP compounds *in vitro* has meaningful impact on post-ischemic injury pathogenesis we tested SA-22 in an *ex vivo* model of IR injury utilizing the Langendorff apparatus. Initial assessment demonstrated that SA-22 had similar cardioprotective effects compared to 19,20-EDP **(Figure 3.2A-D)**. Importantly, pre-ischemic myocardial function was comparable across all treatment groups **(Figure 3.2A-D)**. Hearts perfused with SA-22 had significantly improved postischemic left ventricular developed pressure (LVDP) as well as rates of contraction (dP/dt max) and relaxation (dP/dt min) compared to the vehicle IR group **(Figure 3.2A-D)**. Perfusion with the pan-sirtuin inhibitor NAM as well as the SIRT3-selective inhibitor 3-TYP markedly reduced SA-22-induced recovery of LVDP, dP/dt max, and dP/dt min **(Figure 3.2A-D)**. Lastly, there were no notable differences in heart rate between all study groups **(Figure 3.2E)**. Altogether, these data suggest that SA-22 can improve postischemic functional recovery.

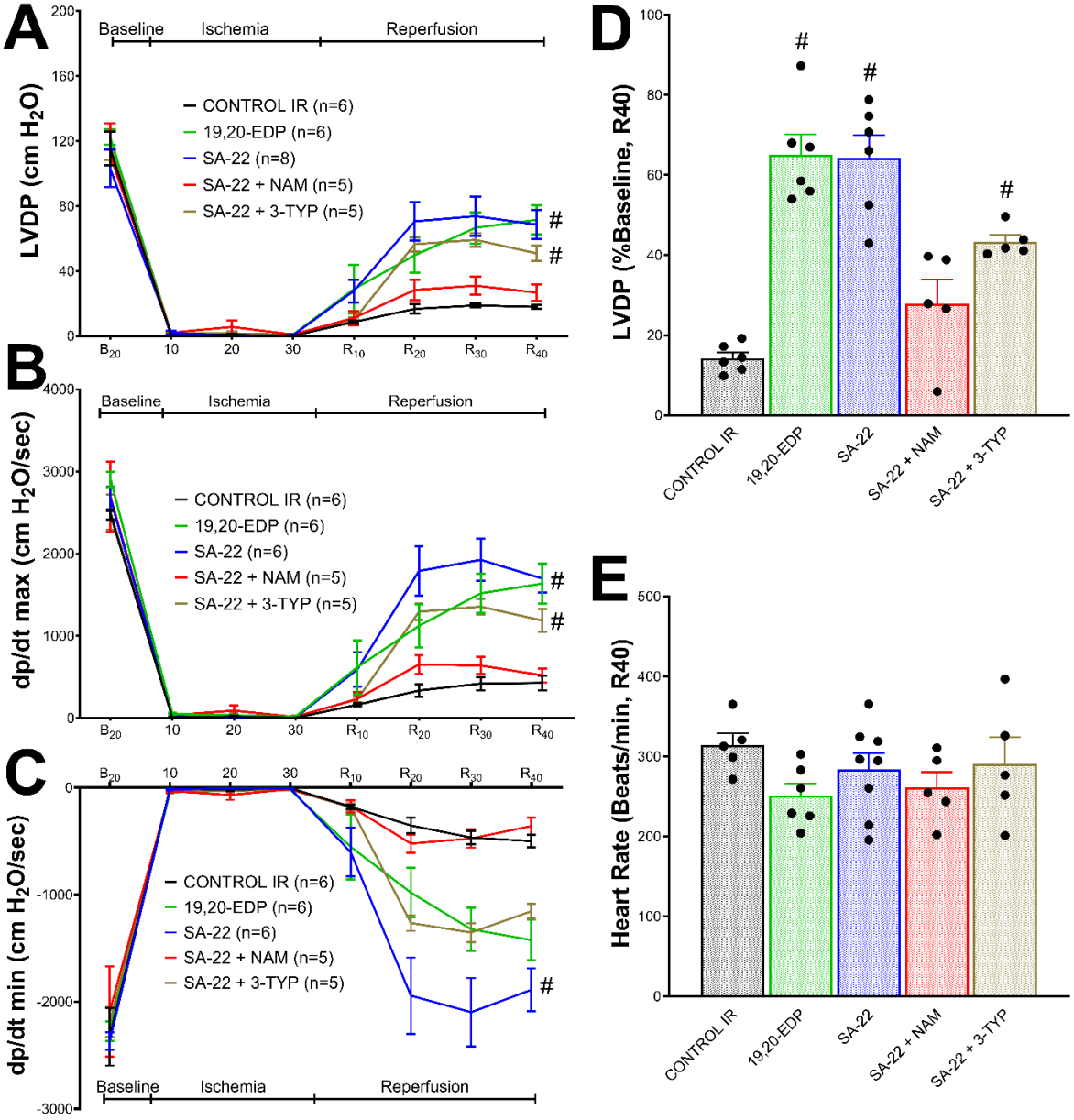

A
Baseline
Ischemia
Reperfusion
LVDP (cm H2O)
CONTROL IR (n=6)
19,20-EDP (n=6)
SA-22 (n=8)
SA-22 + NAM (n=5)
SA-22 + 3-TYP (n=5)
B20
10
20
30
R10
R20
R30
R40
B
dp/dt max (cm H2O/sec)
CONTROL IR (n=6)
19,20-EDP (n=6)
SA-22 (n=6)
SA-22 + NAM (n=5)
SA-22 + 3-TYP (n=5)
C
dp/dt min (cm H2O/sec)
CONTROL IR (n=6)
19,20-EDP (n=6)
SA-22 (n=6)
SA-22 + NAM (n=5)
SA-22 + 3-TYP (n=5)
D
LVDP (%Baseline, R40)
CONTROL IR
19,20-EDP
SA-22
SA-22 + NAM
SA-22 + 3-TYP
E
Heart Rate (Beats/min, R40)
CONTROL IR
19,20-EDP
SA-22
SA-22 + NAM
SA-22 + 3-TYP

Figure 3.2. *SA-22 enhanced postischemic functional recovery.* Hearts were assessed for contractile function at baseline (B_{20}), during ischemia (10, 20, 30), and after reperfusion (R_{10}, R_{20}, R_{30}, and R_{40}). Measurement parameters included LVDP (A,D), rate of contraction (dP/dt max) (B), and rate of relaxation (dP/dt min) (C). Heart rate was assessed as beats per minute (BPM) (E). Treatments; vehicle (0.1% v/v DMSO), 19,20-EDP (1 μM), SA-22 (1 μM), NAM (30 μM), and 3-TYP (50 μM). Values represent mean ± SEM; # *p<0.05 vs. vehicle IR (n = 5-8 per group). LVDP; left ventricular developed pressure. 3-TYP: 3-(1H-1,2,3-triazol-4-yl) pyridine, EDP: Epoxydocosapentaenoic acid, NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid.

3.3 SA-22 preserves respiratory chain function following IR-injury

Preserving mitochondrial respiration is critical to ameliorating myocardial injury in post-myocardial infarction and post-ischemic contexts(393). Furthermore, SIRT3 is a known regulator of myocardial mitochondrial dynamics and respiratory capacity(394). Thus, we assessed the catalytic activity of complex I, II, III, IV, and CS, an aerobic metabolism biomarker due to its role as the rate-limiting factor for Krebs cycle entry(395). IR injury significantly decreased enzymatic activity in complexes I, II, III and IV when compared to the aerobic vehicle group **(Figure 3.3A, B, D, E)**. Perfusion with SA-22 consistently preserved catalytic activity of all 4 complexes following the myocardial insult **(Figure 3.3A, B, D, E)**. Interestingly, NAM co-treatment abrogated analog-induced protection of activity at complexes I and II but not III or IV, suggesting sirtuins are potentially important mediators for the benefits evoked by EpFAs **(Figure 3.3A, B, D, E, G)**. Protein expression levels of succinate dehydrogenase subunit A, a major catalytic subunit of complex II, were unchanged across all groups **(Figure 3.3C)**. Intriguingly, while SA-22 and vehicle IR treatment did not appear to change COX IV expression, treatment with NAM alongside SA-22 significantly reduced protein levels when compared to SA-22 alone **(Figure 3.3F)**. There were no significant differences in CS activity and expression across all treatment groups **(Figure 3.4A, B)**. Interestingly, perfusion with SA-22 partially ameliorated the accumulation of shorter OPA1 isoforms stimulated by IR-injury **(Figure 3.4C)**. Treatment with NAM did not appear to reduce this effect.

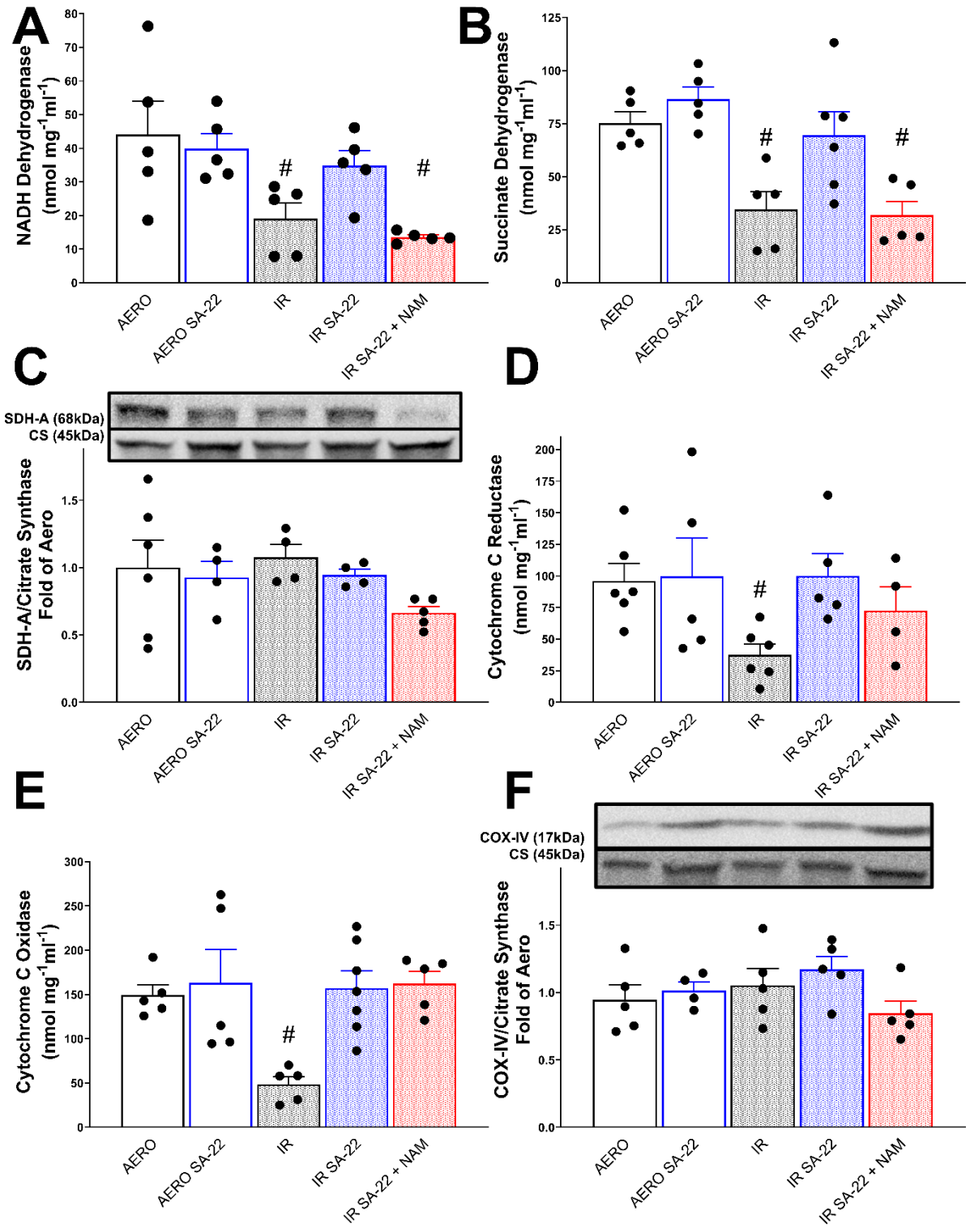

A
NADH Dehydrogenase (nmol mg-1ml-1)
B
Succinate Dehydrogenase (nmol mg-1ml-1)
C
SDH-A (68kDa)
CS (45kDa)
SDH-A/Citrate Synthase Fold of Aero
D
Cytochrome C Reductase (nmol mg-1ml-1)
E
Cytochrome C Oxidase (nmol mg-1ml-1)
F
COX-IV (17kDa)
CS (45kDa)
COX-IV/Citrate Synthase Fold of Aero
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
#

Figure 3.3. *SA-22 treatment preserved ETC function following IR-injury.* Mitochondrial extract from perfused hearts were assessed for ETC activity (nmol/mg/ml) at complex(s) I (A), II (B), III (D), and IV (E). Representative immunoblots and densitometric quantification for SDH-A (C), and COX IV (F). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs. aerobic control (n = 4-6 per group). CS; citrate synthase. ETC; electron transport chain. VDAC; voltage-dependent anion channel. SDH-A; succinate dehydrogenase subunit A. NADH; reduced nicotinamide adenine dinucleotide. COX IV; cytochrome c oxidase. NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid

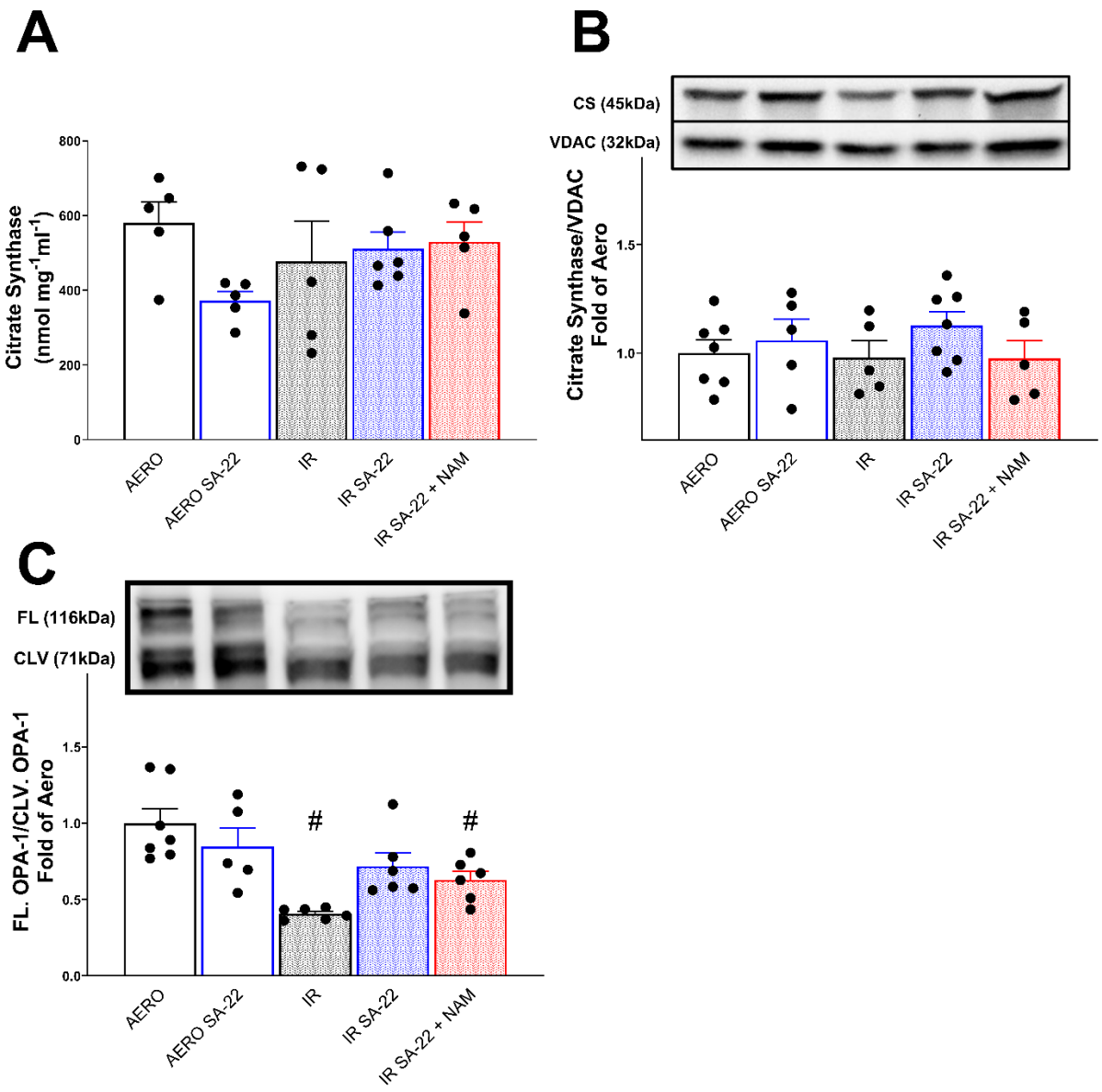

A
Citrate Synthase (nmol mg-1 ml-1)
800
600
400
200
0
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
B
CS (45kDa)
VDAC (32kDa)
Citrate Synthase/VDAC Fold of Aero
1.5
1.0
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
C
FL (116kDa)
CLV (71kDa)
FL. OPA-1/CLV. OPA-1 Fold of Aero
1.5
1.0
0.5
0.0
#
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM

Figure 3.4. *Perfusion with SA-22 protected long OPA1 isoforms.* Mitochondrial extract from perfused hearts were assessed for CS activity and OPA1 cleavage. Enzymatic activity as well as representative immunoblots and densitometric quantification of citrate synthase (A, B). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs. aerobic control (n = 4-6 per group). FL; full-length. CLV; cleaved. OPA1; optic atrophy type-1, VDAC: voltage-dependent anion channel. NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid

3.4 Sirtuin function is maintained in post-IR hearts perfused with SA-22

SIRT3 is capable of deacetylating and activating several targets involved with cellular stress responses, including $MnSOD_2$, whose ability to scavenge ROS is greatly enhanced upon deacetylation by SIRT3(331-333). Importantly, over 90% of the cellular ROS produced during IR injury arises from electrons escaping disrupted ETC subunits, which are known SIRT3 substrates. Thus, we theorized that analogs of 19,20-EDP may limit mitochondrial dysfunction, ROS production, and oxidative damage following IR injury by preserving sirtuin activity(132,143,396,397). From our results, SIRT3 activity was significantly reduced by IR injury when compared to the aerobic vehicle group **(Figure 3.5A)**. However, perfusion with SA-22 significantly attenuated the decline in sirtuin activity caused by IR injury **(Figure 3.5A)**. While SIRT3 protein expression showed no significant differences between treatment groups **(Figure 3.5B)**. The changes in SIRT3 activity were reflected in the acetylation levels of two known SIRT3 substrates, $MnSOD_2$ and lysine residues, where SA-22 prevented an increase in acetylation of both markers induced by IR injury **(Figure 3.5C, D)**. NAM co-perfusion attenuated SA-22-mediated protection of sirtuin activity, reducing SIRT3 activity levels and enhancing acetylation of $MnSOD_2$ and lysine residues **(Figure 3.5C, D)**.

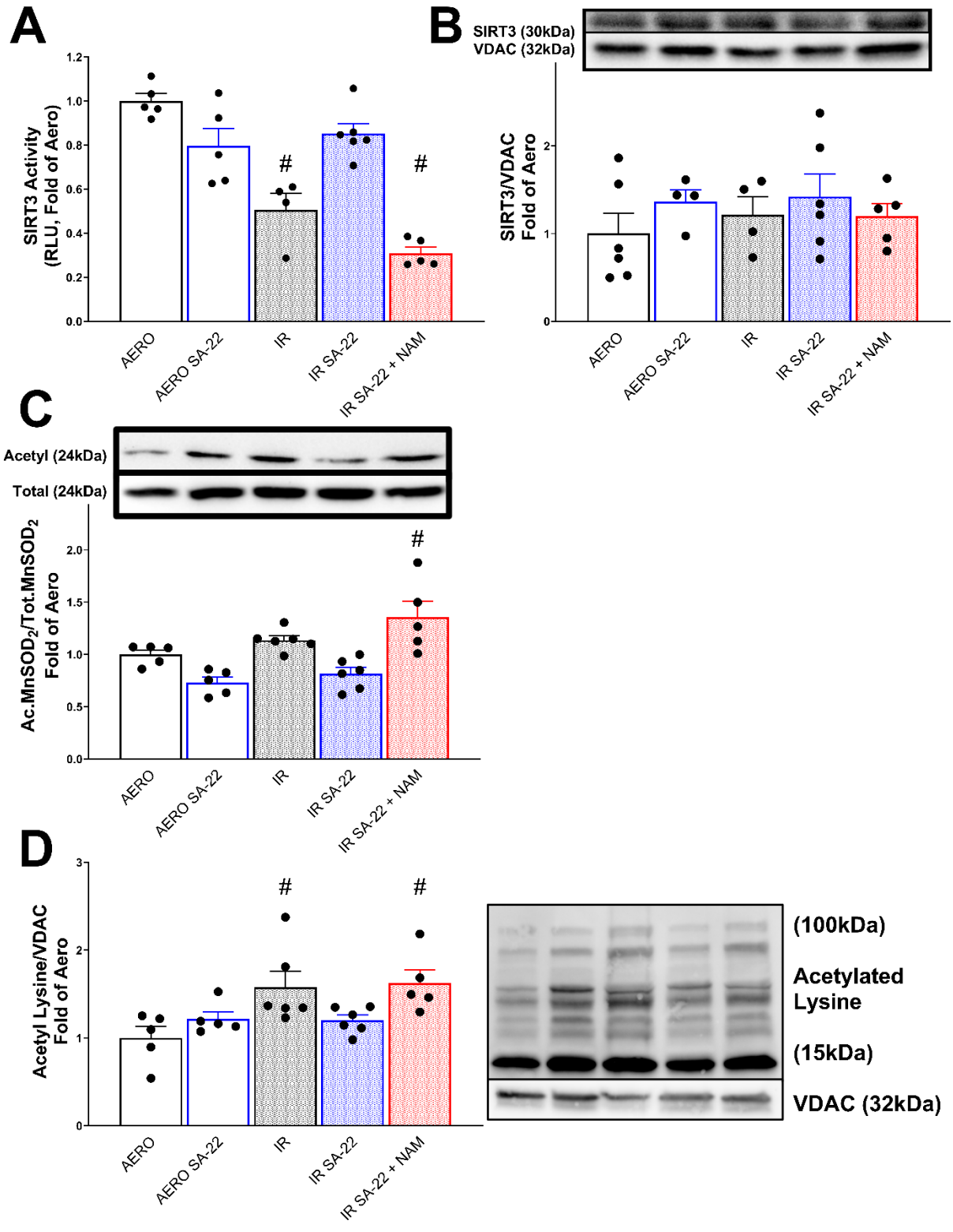

A
SIRT3 Activity (RLU, Fold of Aero)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
B
SIRT3 (30kDa)
VDAC (32kDa)
SIRT3/VDAC Fold of Aero
2
1
0
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
C
Acetyl (24kDa)
Total (24kDa)
Ac.MnSOD2/Tot.MnSOD2 Fold of Aero
2.0
1.5
1.0
0.5
0.0
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
D
Acetyl Lysine/VDAC Fold of Aero
3
2
1
0
#
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
(100kDa)
Acetylated Lysine
(15kDa)
VDAC (32kDa)

Figure 3.5. *SA-22 administration preserved SIRT3 activity against IR injury.* SIRT3 deacetylase activity assayed with fluorescent kit in isolated mitochondrial fractions (A). Representative immunoblots and densitometric quantification of SIRT3 (B), acetyl $MnSOD_2$ (C), and acetyl lysine (D). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs. aerobic control (n = 4-5 per group). AC; acetyl. RLU; relative luminescence units. VDAC; voltage-dependent anion channel. $MnSOD_2$; manganese superoxide dismutase. SIRT3; silent mating type information regulation 2 homolog 3. NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid

In addition to assessing mitochondrial SIRT3 activity post-IR, we also explored potential changes in cytosolic SIRT1 activity. SIRT1 is another member of the sirtuin family of deacetylases that shuttles between the cytosol and nucleus, regulating the transcription of a plethora of genes related to mitochondrial biogenesis via post-translational modification of PGC-1α(351). Similarly to SIRT3, SA-22 protected the catalytic activity of SIRT1 against the deleterious effects of IR injury **(Figure 3.6A)**. There were no significant differences in the protein expression of cytosolic SIRT1 across all treatment groups **(Figure 3.6B)**.

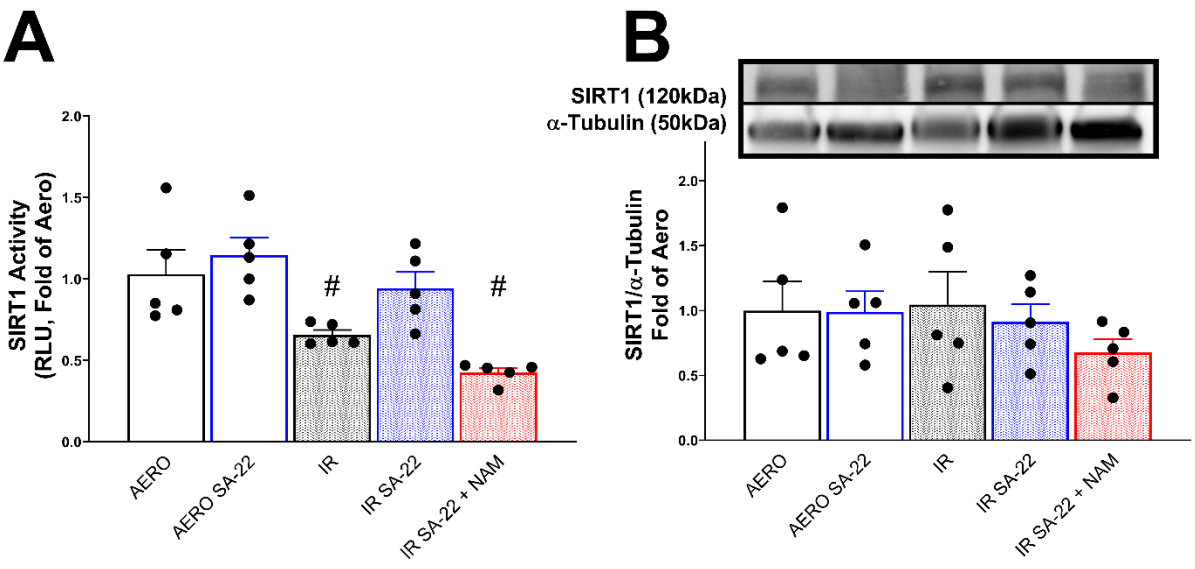


Figure 3.6. *SA-22 administration preserved SIRT1 activity against IR injury.* SIRT1 deacetylase activity assayed with fluorescent kit in isolated cytosolic fractions (A). Representative immunoblots and densitometric quantification of SIRT1 (B). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs. aerobic control (n = 4-5 per group). RLU; relative luminescence units. SIRT1; silent mating type information regulation 2 homolog 1. NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid.

3.5 Perfusion with SA-22 attenuates IR-injury-induced pyroptosis

Caspase-1 is a key mediator of pyroptotic cell death, transducing inflammatory signals by proteolytically cleaving the pyroptotic pore protein gasdermin D (GSDMD) as well as secreted cytokine interleukin 1 beta (IL-1β) following IR injury(43,45,398). We tested SA-22 for potential attenuation of pyroptosis. Caspase-1 activity levels were significantly increased in the vehicle IR group compared to aerobic control, with SA-22 treatment returning them to aerobic vehicle levels **(Figure 3.7A)**. Secondly, SA-22 reduced cleavage of IL-1β when compared to vehicle IR, where virtually all IL-1β was cleaved/active **(Figure 3.7B)**. Once activated via proteolytic cleavage, GSDMD localizes to the cellular plasma membrane (PM) where it functions as a pore for pyroptotic signals such as IL-1β to be secreted(399). Across all treatment groups, only the PMs of the vehicle IR hearts possessed any notable levels of cleaved GSDMD. SA-22 administration reduced the PM-localization of cleaved GSDMD **(Figure 3.7C)**. Unexpectedly, despite the ability of NAM to prevent the benefits induced by SA-22 regarding myocardial function and respiration, there was no effect on the activation of pyroptosis when compared to treatment with SA-22 alone **(Figure 3.7A-C)**. Cumulatively, these data suggest that maintenance of sirtuin activity is important to the cardioprotective actions exerted by EpFAs in the context of myocardial IR injury.

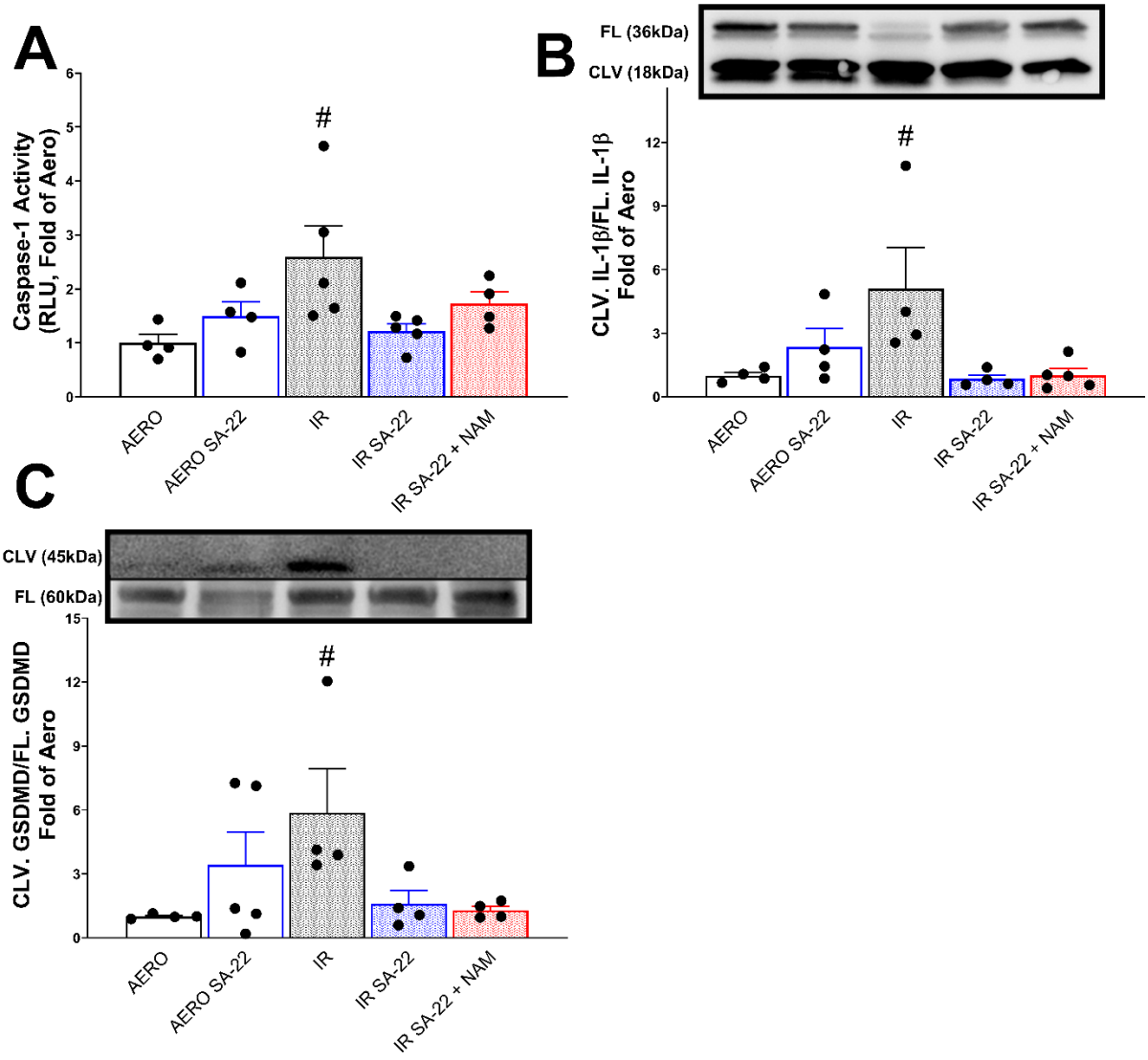

A
Caspase-1 Activity (RLU, Fold of Aero)
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
B
FL (36kDa)
CLV (18kDa)
CLV. IL-1β/FL. IL-1β Fold of Aero
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM
C
CLV (45kDa)
FL (60kDa)
CLV. GSDMD/FL. GSDMD Fold of Aero
#
AERO
AERO SA-22
IR
IR SA-22
IR SA-22 + NAM

Figure 3.7. *SA-22 perfusion attenuated IR-induced pyroptosis.* Caspase-1 proteolytic activity assayed with a fluorescent kit in isolated cytosol fraction (A). Representative immunoblots and densitometric quantification of cytosolic cleaved IL-1β normalized to non-cleaved IL-1β (B), and cleaved GSDMD resolubilized from plasma membranes (C). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs. aerobic control (n = 4-5 per group). FL; full-length. CLV; cleaved. RLU; relative luminescence units. IL-1β; interleukin-1 beta. GSDMD; gasdermin D. NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid.

3.6 SA-22 protects myocardial cells against HR injury

Previous work has demonstrated EDPs are protective against HR injury in an atrial cell line, HL-1 cardiac cells(243). In the current study, we employed the ventricular cardiac myoblast cell line H9c2 to assess the cytoprotective effect of SA-22. First, successful differentiation of H9c2 cells using retinoic acid (RA) was confirmed by densitometric blot analysis of cardiac troponin T levels (cTnT) and phase contrast microscopy compared to cells that did not receive RA **(Figure 3.8)**.

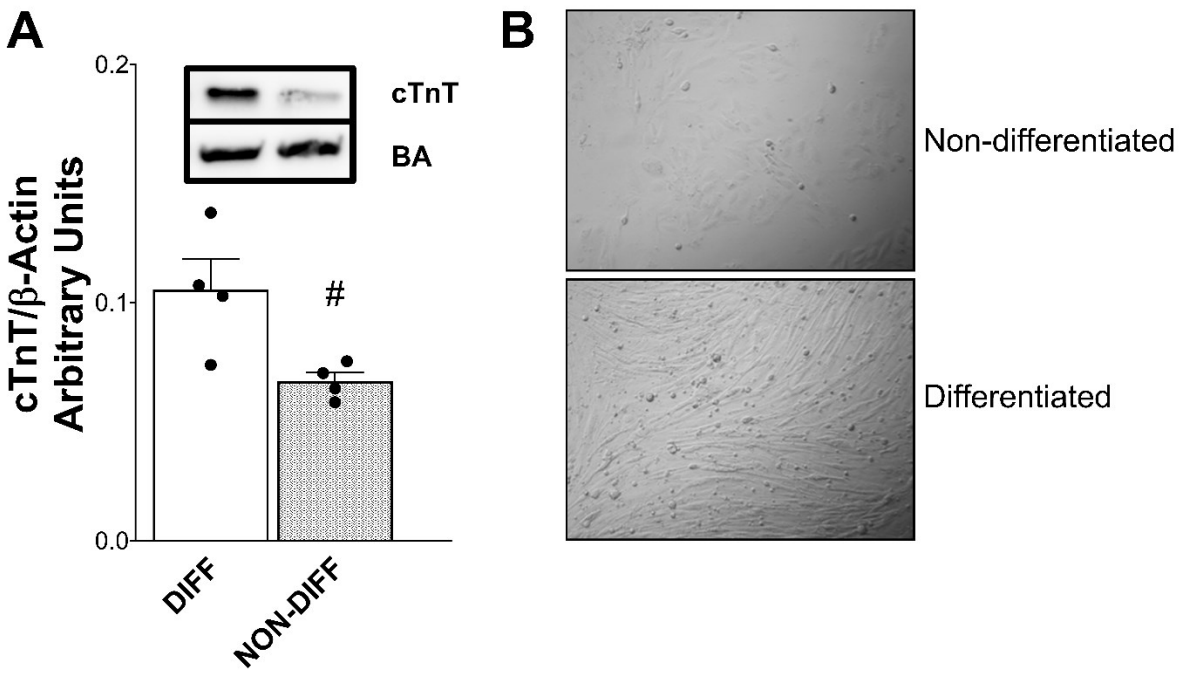


Figure 3.8. *Validation of RA-stimulated cellular differentiation.* Retinoic acid-induced cell differentiation was confirmed by immunoblotting and densitometric quantification of cTnT normalized to β-actin (A). Representative phase contrast microscope images (B). Treatments; RA (10 μM). Values represent mean ± SEM, **p*<0.05 vs DIFF control, #*p*<0.05 vs DIFF (n = 4 per group). BA; Beta-actin. cTnT; cardiac Troponin T. DIFF; Differentiated. RA; Retinoic acid.

Exposing H9c2 cells to HR injury caused a significant decrease in cell viability, assessed by Trypan Blue exclusion assay and a fluorogenic 20S Proteasome activity assay **(Figure 3.9A, B)**. Notably, SA-22 treatment attenuated a significant increase in caspase-1 activity elicited by HR **(Figure 3.9C)**. NAM treatment prevented SA-22 from limiting caspase-1 activity, suggesting that sirtuins have a role in the cytoprotective response. HR injury also stimulated robust increases in acetylation of $MnSOD_2$, indicating a reduction in SIRT3 activity, which was reversed by addition of SA-22 **(Figure 3.9D)**. SIRT1 levels were relatively unchanged across treatment groups **(Figure 3.9E)**. HR cells also possessed a marked reduction in SIRT3 levels compared to aerobic baseline, which SA-22 treatment rescued **(Figure 3.9F)**. Notably, NAM treatment did not affect SIRT levels **(Figure 3.9E, F)**.

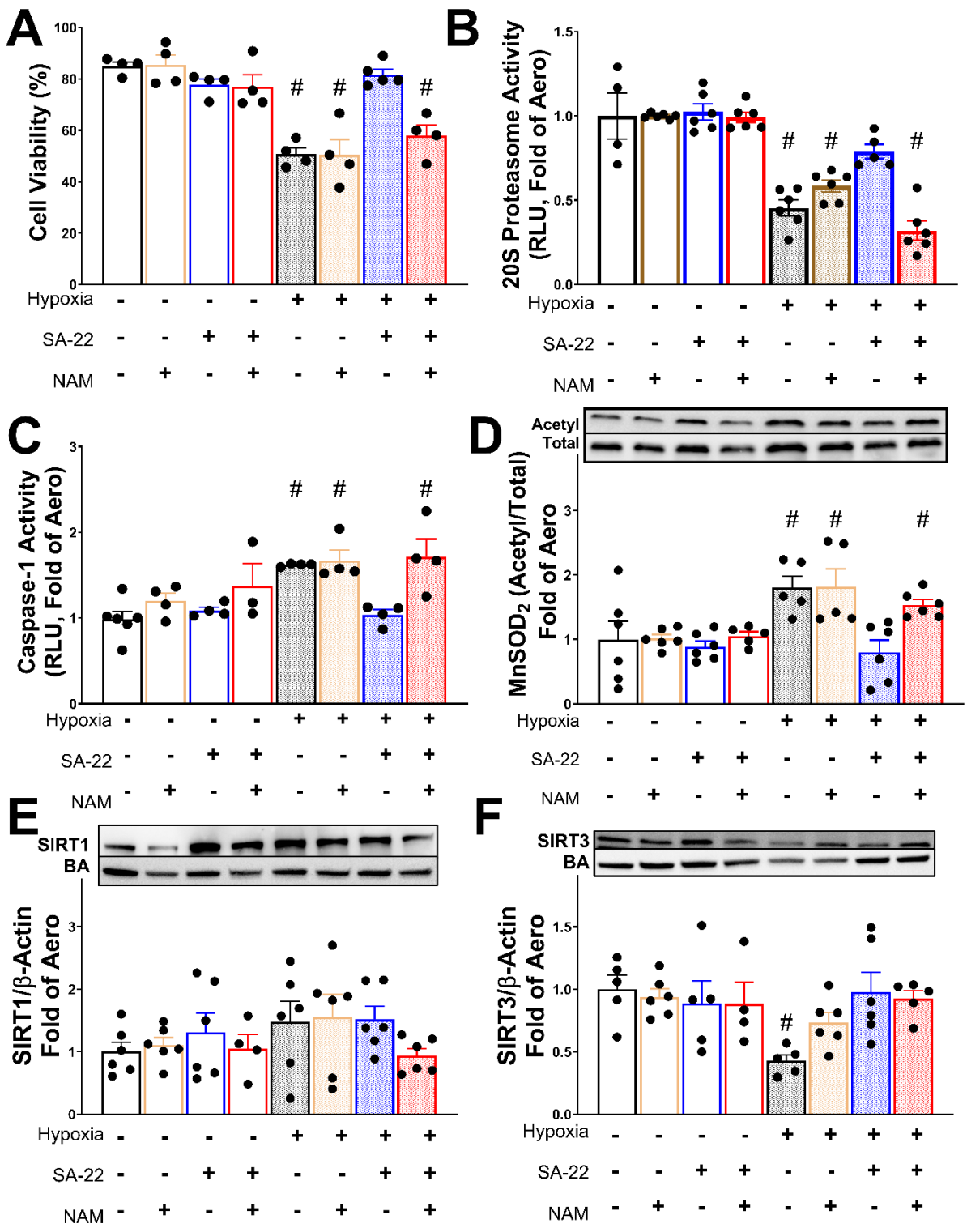

A
Cell Viability (%)
100
80
60
40
20
0
#
#
#
Hypoxia - - - - + + + +
SA-22 - - + + - - + +
NAM - + - + - + - +
B
20S Proteasome Activity (RLU, Fold of Aero)
1.5
1.0
0.5
0.0
#
#
#
Hypoxia - - - - + + + +
SA-22 - - + + - - + +
NAM - + - + - + - +
C
Caspase-1 Activity (RLU, Fold of Aero)
3
2
1
0
#
#
#
Hypoxia - - - - + + + +
SA-22 - - + + - - + +
NAM - + - + - + - +
D
Acetyl
Total
MnSOD2 (Acetyl/Total) Fold of Aero
3
2
1
0
#
#
#
Hypoxia - - - - + + + +
SA-22 - - + + - - + +
NAM - + - + - + - +
E
SIRT1
BA
SIRT1/β-Actin Fold of Aero
3
2
1
0
Hypoxia - - - - + + + +
SA-22 - - + + - - + +
NAM - + - + - + - +
F
SIRT3
BA
SIRT3/β-Actin Fold of Aero
1.5
1.0
0.5
0.0
#
Hypoxia - - - - + + + +
SA-22 - - + + - - + +
NAM - + - + - + - +

Figure 3.9. *SA-22 ameliorated HR injury in H9c2 cells.* General cell viability after 24 hours of hypoxia followed by 6 hours of reoxygenation was assayed via trypan blue cell counting (A) and fluorescent cytosolic 20S proteasome activity kit (B). Caspase-1 proteolytic activity assayed with a fluorescent kit (C). Representative immunoblots and densitometric quantification of acetyl $MnSOD_2$ (D), SIRT1 (E), and SIRT3 (F). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs aerobic vehicle (n = 3-6 per group). BA; Beta-actin. $MnSOD_2$; manganese superoxide dismutase. SIRT1/3; silent mating type information regulation 2 homolog 1/3. NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. RLU; relative luminescence units.

To further validate and support the evidence of cytoprotection observed in our H9c2 cell model, we also employed the use of primary NRCMs as an *in vitro* model of myocardial HR injury. Similarly to the results observed in our H9c2 cells, both 19,20-EDP and SA-22 protected NRCM cell viability against HR injury as assessed via 20S Proteasome assay **(Figure 3.10A)**. Additionally, SA-22 and native 19,20-EDP abrogated the induction of caspase-1 activity following experimental insult **(Figure 3.10B)**.

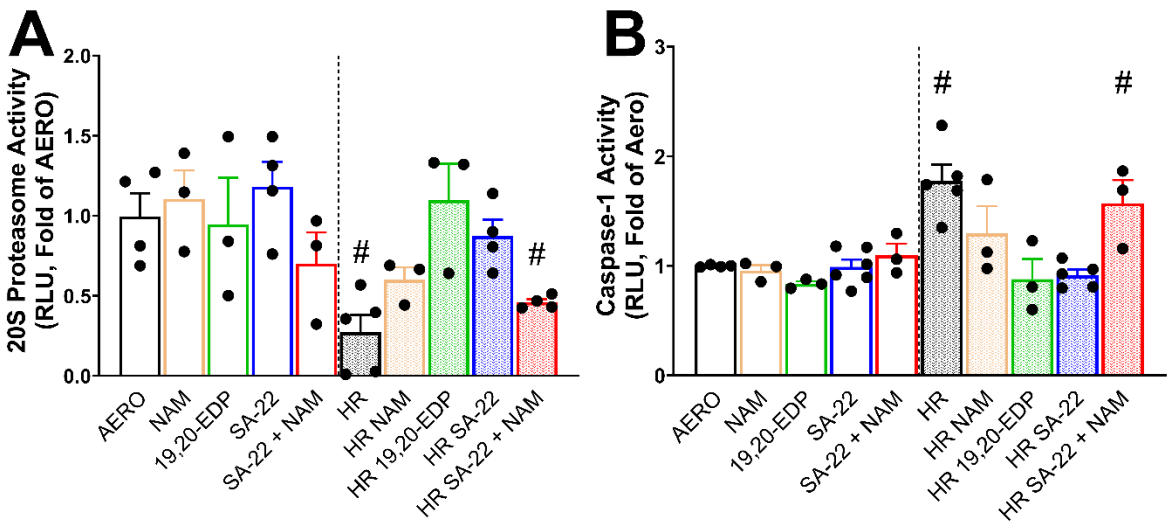


Figure 3.10. *SA-22 ameliorated HR injury in NRCMs.* General cell viability after 24 hours of hypoxia followed by 6 hours of reoxygenation was assayed via fluorescent cytosolic 20S proteasome activity kit (A). Caspase-1 proteolytic activity assayed with a fluorescent kit (B). Treatments; vehicle (0.1% v/v DMSO), 19,20-EDP (1 μM), SA-22 (1 μM), and NAM (30 μM). Values represent mean ± SEM, # $p<0.05$ vs aerobic vehicle (n = 3-6 per group). NAM; Nicotinamide, SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. RLU; relative luminescence units. EDP; Epoxydocosapentaenoic acid. HR; hypoxia-reoxygenation.

3.7 SA-22 preserves mitophagic flux post-HR injury

Following observations that SA-22 preserves ETC activity and ameliorates OPA1 cleavage following IR challenge ex vivo, we wanted to explore the potential impact of SA-22 and SIRT3 potentiation on myocardial mitochondrial homeostasis following HR injury *in vitro*. To that end, we wondered if the mechanism of action (MoA) for SA-22's cardioprotective properties involved modulation of mitophagy. To confirm whether SA-22 triggers a mitophagic response irrespective of cellular injury, we treated non-challenged H9c2 cells with SA-22 and harvested them at several timepoints (30-minutes, 1-hour, and 2-hours). FCCP was used a positive control for stimulation of mitophagy(400). Western blot analysis of heavy membrane-enriched fractions demonstrated that SA-22 did not significantly trigger the mitochondrial-localization of several markers associated with mitophagy such as dynamin-related protein-1 (DRP-1), PTEN-induced kinase-1 (PINK1), p62, and Microtubule-associated proteins 1A/1B light chain 3B (LC3B) **(Figure 3.11A, B, D, E**) (401-405). Interestingly, SA-22 treatment appears to cause significant mitochondrial localization of Parkin, independent of any of the other assessed markers **(Figure 3.11C)**.

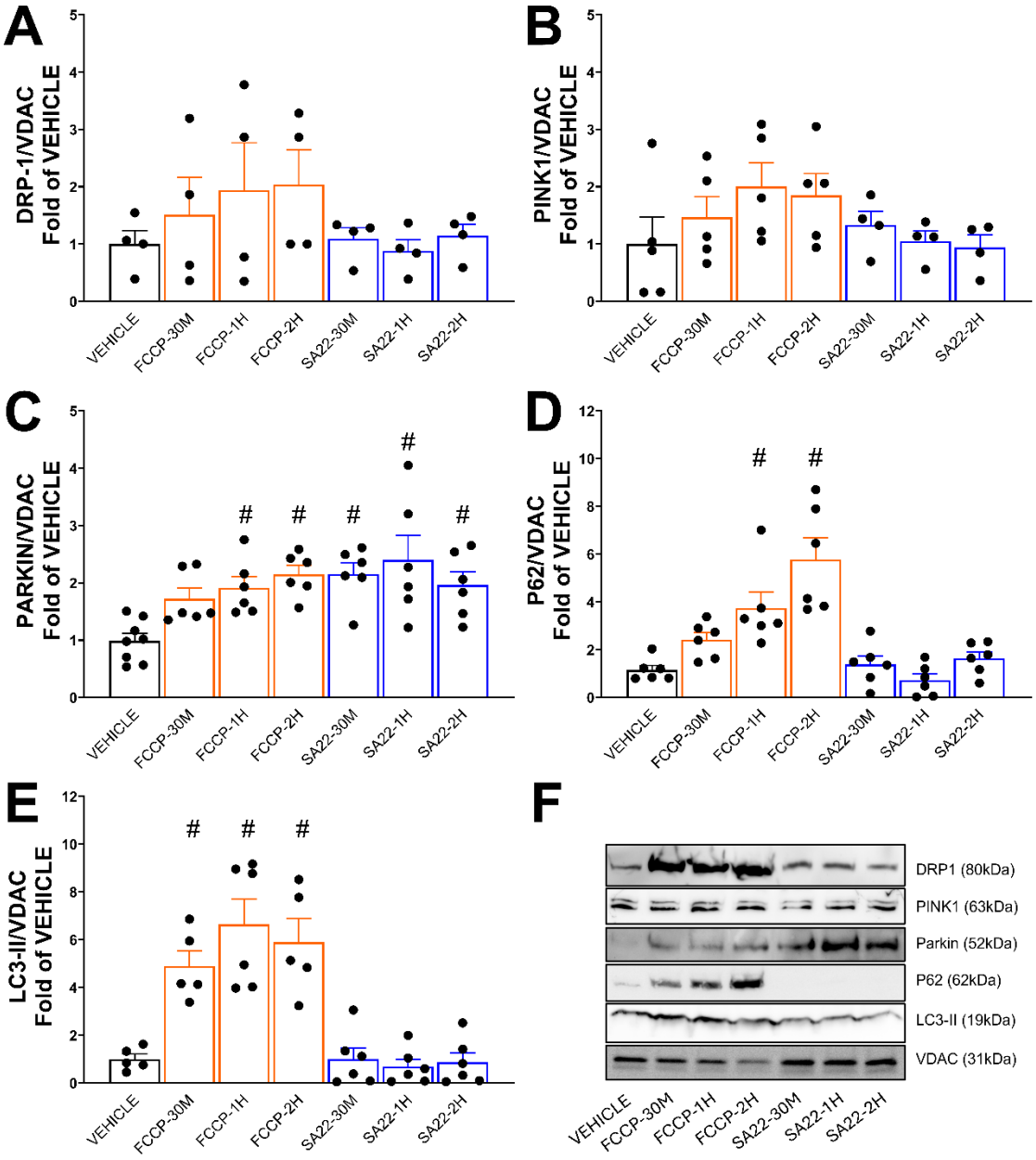

A
DRP-1/VDAC
Fold of VEHICLE
B
PINK1/VDAC
Fold of VEHICLE
C
PARKIN/VDAC
Fold of VEHICLE
D
P62/VDAC
Fold of VEHICLE
E
LC3-II/VDAC
Fold of VEHICLE
F
VEHICLE
FCCP-30M
FCCP-1H
FCCP-2H
SA22-30M
SA22-1H
SA22-2H
DRP1 (80kDa)
PINK1 (63kDa)
Parkin (52kDa)
P62 (62kDa)
LC3-II (19kDa)
VDAC (31kDa)

Figure 3.11. *SA-22 does not stimulate mitophagy in the absence of cellular injury*. Heavy membrane-enriched fractions from H9c2 cell lysates were assessed for mitochondrially-localized mitophagy markers via western blotting. Densitometric quantification for DRP-1 (A), PINK1 (B), Parkin (C), p62 (D), and LC3-II (E). Representative immunoblots for mitophagy markers (F). Treatments; vehicle (0.1% v/v DMSO), FCCP (10 μM), SA-22 (1 μM). Values represent mean ± SEM, # $p<0.05$ vs aerobic vehicle (n = 4-8 per group). DRP-1; dynamin-related protein-1. VDAC: voltage-dependent anion channel. FCCP; Carbonyl cyanide p-trifluoromethoxyphenylhydrazone. PINK1; PTEN-induced kinase-1. P62; sequestosome-1. Microtubule-associated proteins 1A/1B light chain 3. SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid.

While the stimulation of mitophagy by hypoxia and its benefits have been well-documented (406), several of the adverse outcomes also associated with HR injury (such as ROS, and NLRP3 inflammasome activation) have been demonstrated to be potent inhibitors of mitophagy(407,408). Thus, we sought to determine if SA-22 could preserve protective mitophagic flux in myocardial cells after HR injury. However, since mitophagy is a highly dynamic and time-dependent process, we opted to employ the use of H9c2 cells stably expressing mito-Keima, a mitochondrially-targeted, lysosome-resistant, fluorescent protein with pH-dependent excitation spectra(409). Use of mito-Keima enables the real-time differentiation between mitochondria present in a neutral pH environment and those within an acidic pH environment (i.e. Autophagosomes/autophagolysosomes) **(Figure 3.12)**. Using ImageJ and Hoechst 33342, we quantified the number of acidic puncta within the mitochondrium and normalized it to the total number of nuclei present in the image field. Immediately following reoxygenation, all hypoxic groups possessed an elevated number of 'mitophagosomes' **(Figure 3.13A)**. Interestingly, by 1-hour post-reoxygenation, both the 19,20-EDP and SA-22 treated groups had significantly fewer mitophagosomes compared to the HR vehicle group **(Figure 3.13B, C)**. At the end of the 6-hour reoxygenation period, mitophagosome levels in the EDP-treated groups had returned to near aerobic levels **(Figure 3.13F, 3.14)**. In contrast, the groups that received co-treatment of 19,20-EDP or SA-22 with a sirtuin inhibitor (NAM or 3-TYP), showed no improvement in the number of mitophagosomes, implying that SIRT activity is vital to the resolution/preservation of functional mitophagic flux following HR injury **(Figure 3.13A-F, 3.14)**. Neither 19,20-EDP or SA-22 appeared to

trigger mitophagic flux in the absence of HR insult, further supporting our initial western blot analysis **(Figure 3.13A-F)**.

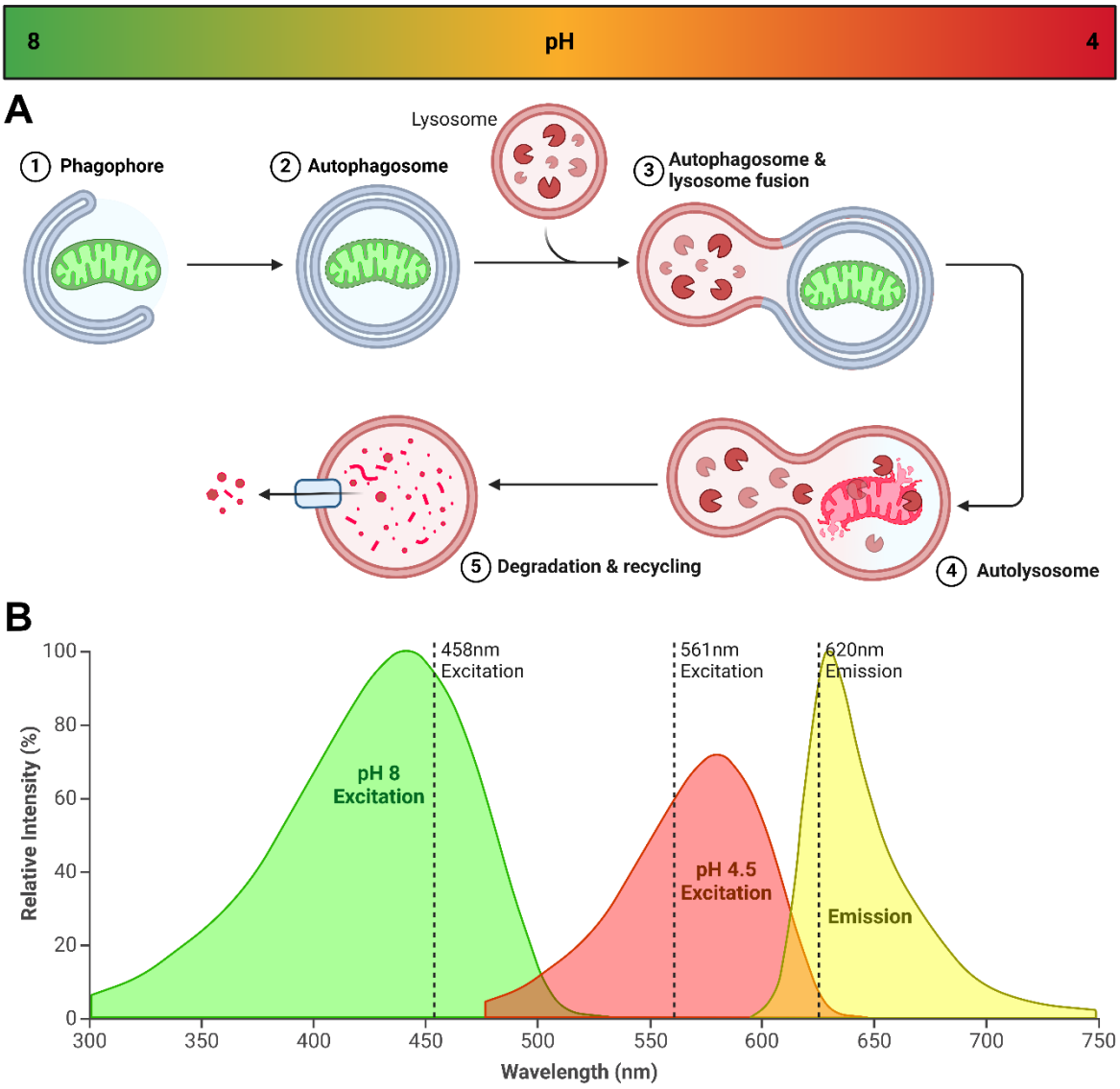


Figure 3.12. *Mito-Keima: A pH-dependent dual-excitation protein fluorophore.* Graphical summary of the pH-dependent change in mito-Keima fluorescence during mitophagy following acidification of the mitochondria by lysosomes **(A)**. Excitation and emission of mitochondrially-targeted Keima fluorescence. The excitation wavelength of the Keima protein is determined by its environmental pH, with an excitation spectrum at neutral pH centered at 440nm, an excitation spectrum at acidic pH centered at 586nm. The emission wavelength for mito-Keima at either pH is approximately 620nm **(B)**.

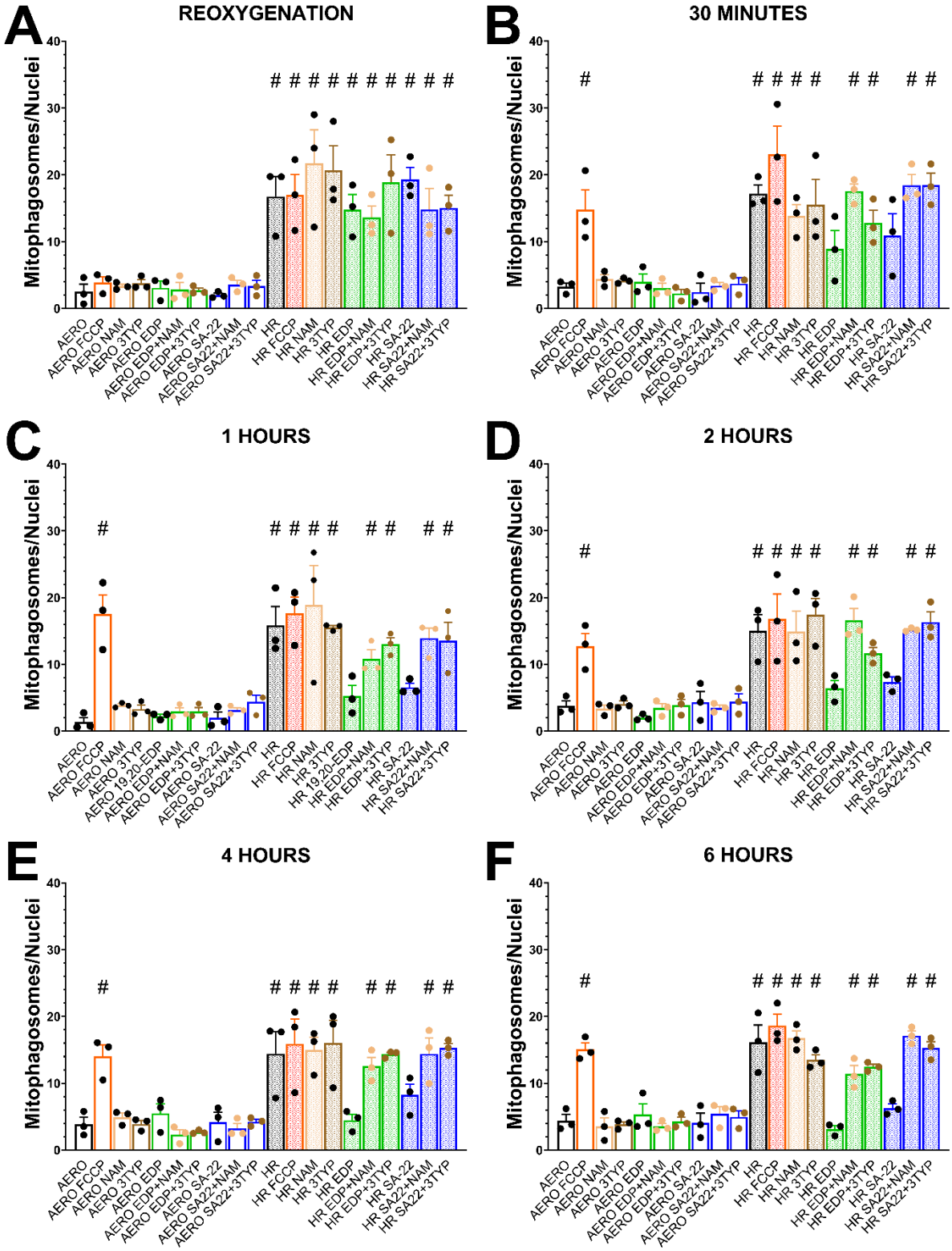

A
REOXYGENATION
B
30 MINUTES
C
1 HOURS
D
2 HOURS
E
4 HOURS
F
6 HOURS
Mitophagosomes/Nuclei
AERO
AERO FCCP
AERO NAM
AERO 3TYP
AERO 19,20-EDP
AERO EDP
AERO EDP+NAM
AERO EDP+3TYP
AERO SA-22
AERO SA22+NAM
AERO SA22+3TYP
HR
HR FCCP
HR NAM
HR 3TYP
HR 19,20-EDP
HR EDP
HR EDP+NAM
HR EDP+3TYP
HR SA-22
HR SA22+NAM
HR SA22+3TYP

Figure 3.13. *SA-22 rescues mitophagy in H9c2 cells following HR injury.* H9c2 cells stably expressing mito-Keima were assessed after treatment with either vehicle, 19,20-EDP, SA-22, or co-treatment of either compound with either NAM, or 3-TYP. Mitophagosome density was normalized to nuclei count at several timepoints post-reoxygenation (Average of 5 random fields' whole-field fluorescence represents each data-point); reoxygenation (A), 30-minutes (B), 1-hour (C), 2-hours (D), 4-hours (E), and 6-hours (F). Treatments; vehicle (0.1% v/v DMSO), FCCP (10 μM), 19,20-EDP (1 μM), NAM (30 μM), 3-TYP (50 μM), or SA-22 (1 μM). Values represent mean ± SEM, # $p<0.05$ vs aerobic vehicle (n = 3 per group). SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. EDP; Epoxydocosapentaenoic acid. 3-TYP: 3-(1H-1,2,3-triazol-4-yl) pyridine. NAM; Nicotinamide. HR; hypoxia-reoxygenation. FCCP; Carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

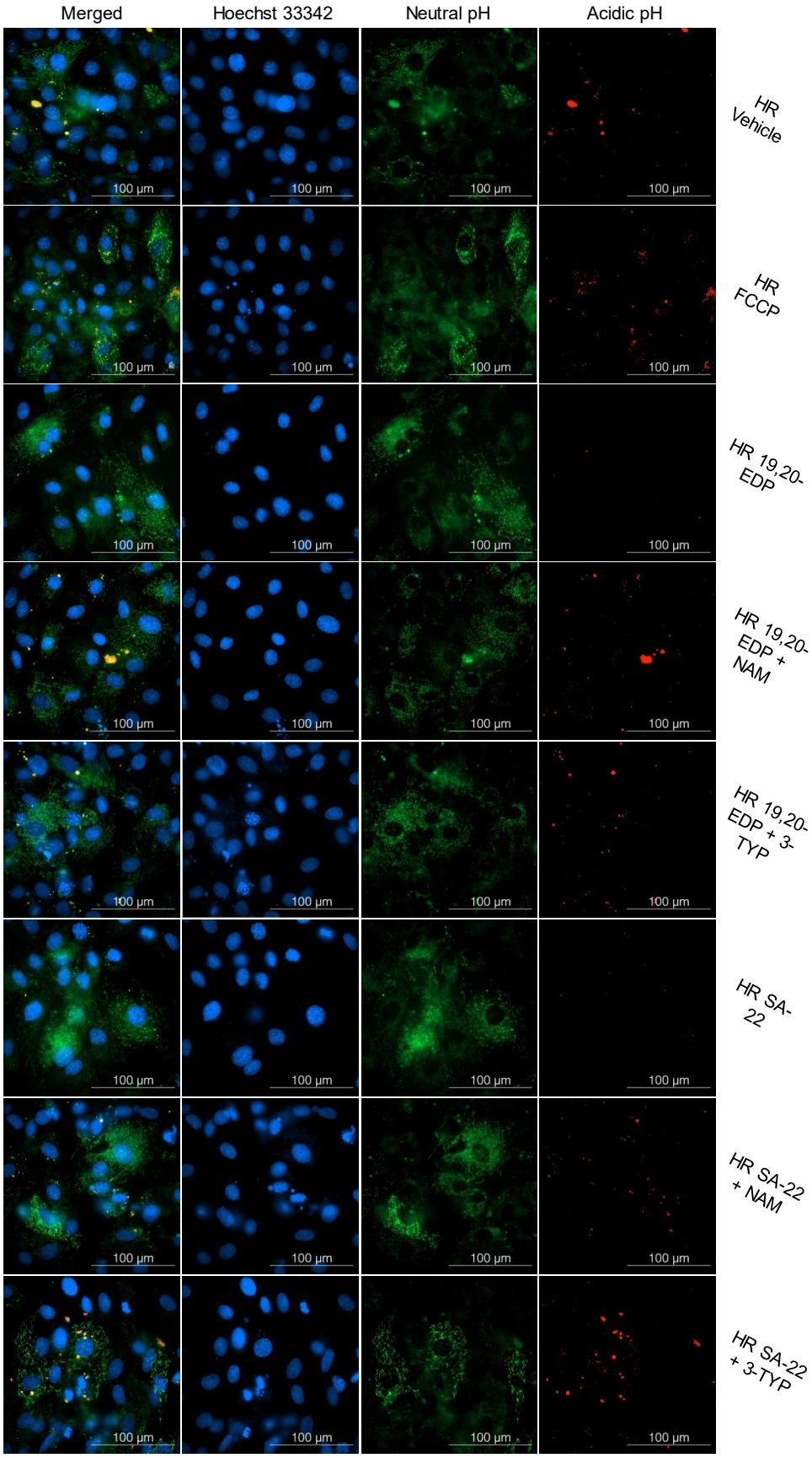

Merged
Hoechst 33342
Neutral pH
Acidic pH
HR Vehicle
HR FCCP
HR 19,20-EDP
HR 19,20-EDP + NAM
HR 19,20-EDP + 3-TYP
HR SA-22
HR SA-22 + NAM
HR SA-22 + 3-TYP
100 µm

Figure 3.14. *Preservation of mitophagy post-HR by EDP compounds is SIRT-dependent.* Representative fluorescence images from H9c2 cells stably expressing mito-Keima were treated with either vehicle, FCCP, 19,20-EDP, SA-22, or co-treatment of either EpFA with either NAM, or 3-TYP. Photos were taken 6-hours post-reoxygenation. White bar = 50 µm. Treatments; vehicle (0.1% v/v DMSO), FCCP (10 µM), 19,20-EDP (1 µM), NAM (30 µM), 3-TYP (50 µM), or SA-22 (1 µM). SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. EDP; Epoxydocosapentaenoic acid. 3-TYP: 3-(1H-1,2,3-triazol-4-yl) pyridine. NAM; Nicotinamide. HR; hypoxia-reoxygenation. FCCP; Carbonyl cyanide p-trifluoromethoxyphenylhydrazone. EpFA; epoxy fatty acid.

To gain further insight into the changes in mitophagy occurring post-reoxygenation, we harvested treated cells after 6-hours of reoxygenation and subjected them to western blot analysis. Hypoxic cells exhibited a significantly increased amount of mitochondrially-localized DRP-1, suggesting that damaged mitochondria were being targeted for removal **(Figure 3.15A)**. Intriguingly, other markers that represent the progression and execution of mitophagy such as Parkin and p62 were significantly decreased among hypoxic groups **(Figure 3.15B-F)**. Importantly, SA-22 treatment significantly ameliorated the deficient localization of Parkin and p62 to mitochondria in a SIRT-dependent manner **(Figure 3.15C, D)**. SA-22 did not appear to influence mitochondrial-localization of PINK1 and LC3B **(Figure 3.15B, E)**.

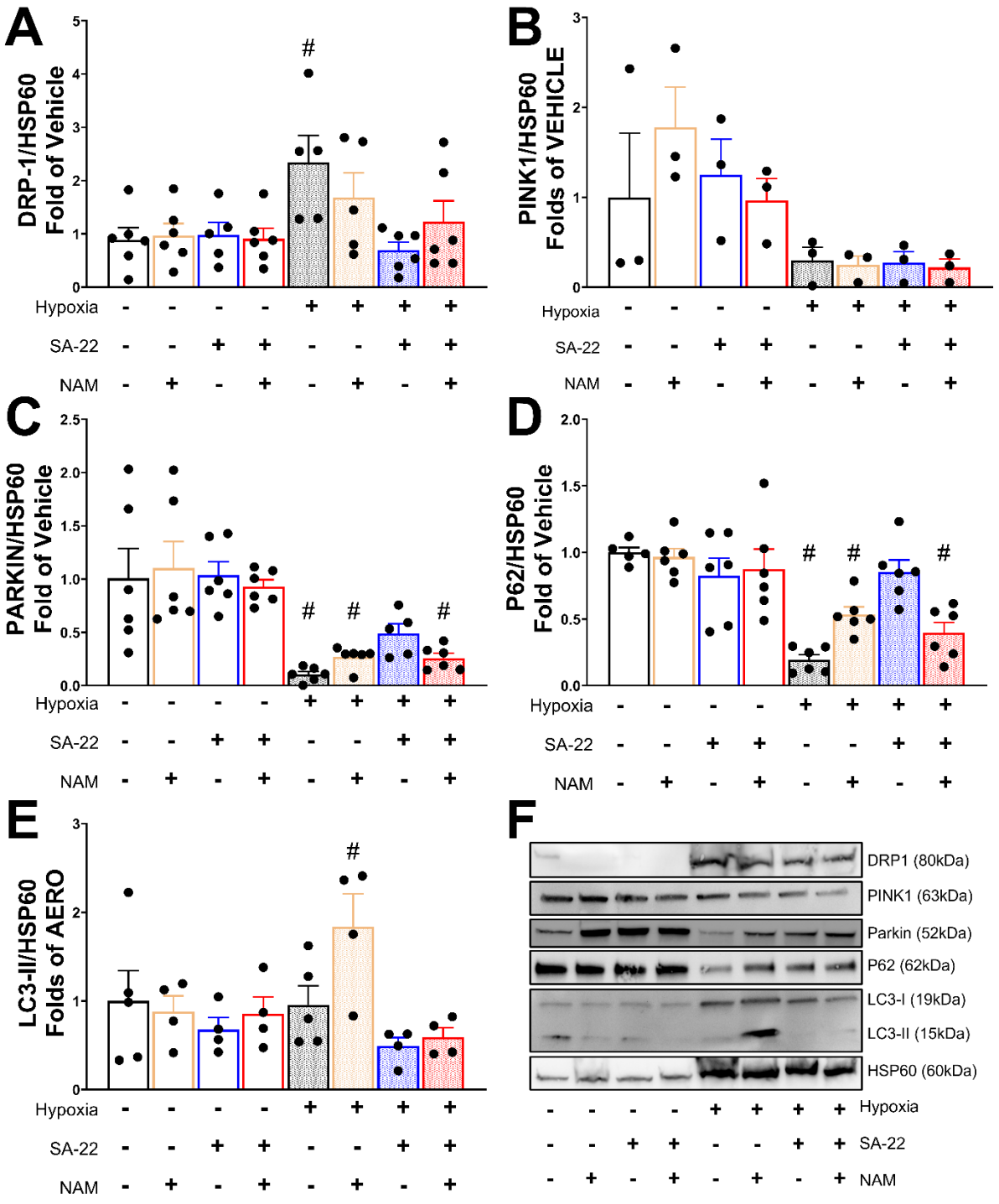

A
DRP-1/HSP60
Fold of Vehicle
B
PINK1/HSP60
Folds of VEHICLE
C
PARKIN/HSP60
Fold of Vehicle
D
P62/HSP60
Fold of Vehicle
E
LC3-II/HSP60
Folds of AERO
F
DRP1 (80kDa)
PINK1 (63kDa)
Parkin (52kDa)
P62 (62kDa)
LC3-I (19kDa)
LC3-II (15kDa)
HSP60 (60kDa)
Hypoxia
SA-22
NAM

Figure 3.15. *SA-22 affects post-HR localization of several mitophagy proteins.* Heavy membrane-enriched fractions from H9c2 cell lysates 6-hours post-reoxygenation were assessed for mitochondrially-localized mitophagy markers via western blotting. Treatments; vehicle (0.1% v/v DMSO), 19,20-EDP (1 μM), NAM (30 μM), 3-TYP (50 μM), or SA-22 (1 μM). Densitometric quantification for DRP-1 (A), PINK1 (B), Parkin (C), p62 (D), and LC3-II (E). Representative immunoblots for mitophagy markers (F). Values represent mean ± SEM, # $p<0.05$ vs aerobic vehicle (n = 4-6 per group). DRP-1; dynamin-related protein-1. VDAC: voltage-dependent anion channel. FCCP; Carbonyl cyanide p-trifluoromethoxyphenylhydrazone. PINK1; PTEN-induced kinase-1. P62; sequestosome-1. Microtubule-associated proteins 1A/1B light chain 3. SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. NAM; Nicotinamide. HR; hypoxia-reoxygenation. 3-TYP: 3-(1H-1,2,3-triazol-4-yl) pyridine.

3.8 SA-22 protects cellular respiratory capacity and ETC efficiency

After demonstrating that the physical relationship between SA-22 and SIRT3 has beneficial effects on the clearance of damaged mitochondria following reoxygenation, we assumed that such effects would be reflected in mitochondrial respiration. Thus, we employed the Oroboros O2k high-resolution respirometer to assess oxygen consumption by H9c2 cells following hypoxia-reoxygenation. Immediately, HR injury significantly reduced basal oxygen consumption (physiological respiration coupled to ATP production) compared to aerobic vehicle control **(Table 3.1)**. Furthermore, HR challenge significantly reduced spare respiratory capacity and total OXPHOS capacity, suggesting ETC impairment **(Figure 3.16A, B)**. SA-22 addition protected cells against HR-induced decreases in sprare respiratory capacity (SRC) and OXPHOS function caused by HR **(Figure 3.16A, B)**. When normalized to total ETC capacity, HR vehicle-treated cells displayed elevated levels of LEAK respiration, implying that a greater proportion of their respiratory capacity is dedicated to compensating for proton and electron leakage, suggesting disruption of the ETC complexes and of the mitochondrial matrix **(Figure 3.16C)**; This was also ameliorated by SA-22. Residual oxygen consumption (ROX) is a total measure of the oxidative side reactions occurring in cells not coupled to ATP production(410). While this may be an effect of an overall decreased respiratory capacity, HR significantly increased ROX **(Figure 3.16D)**.

State	AERO	AERO SA-22	HR VEH-0H	HR VEH-3H	HR VEH-6H	HR SA-22-0H	HR SA-22-3H	HR SA-22-6H
Basal	49.24 ± 0.93	56.85 ± 1.95	21.98 ± 2.67	24.29 ± 2.25	25.14 ± 4.17	53.81 ± 2.45	58.01 ± 2.00	45.83 ± 3.12
PM_L (N_L)	18.67 ± 2.74	24.49 ± 1.88	11.11 ± 1.64	15.74 ± 2.70	14.16 ± 1.93	22.67 ± 1.50	15.12 ± 3.02	25.34 ± 1.24
PM_P (N_P)	49.37 ± 4.94	74.41 ± 6.24	20.11 ± 4.30	32.72 ± 3.88	28.53 ± 4.35	69.63 ± 4.41	44.18 ± 8.17	74.78 ± 6.24
PMc_P (N_P)	70.19 ± 7.25	100.68 ± 8.29	47.77 ± 11.98	48.28 ± 6.77	40.32 ± 4.95	84.52 ± 3.84	54.79 ± 9.40	78.31 ± 5.92
PMG_P (N_P)	70.06 ± 7.06	100.14 ± 8.53	35.40 ± 10.74	45.39 ± 6.39	48.85 ± 3.86	84.41 ± 4.83	80.65 ± 15.89	83.28 ± 11.16
$PMGS_P$ (NS_P)	116.91 ± 10.18	177.49 ± 6.81	70.31 ± 17.12	53.63 ± 7.11	80.87 ± 6.20	198.73 ± 20.21	198.83 ± 41.88	175.23 ± 11.75
$PMGS_E$ (NS_E)	178.21 ± 13.43	227.79 ± 27.75	79.06 ± 21.26	103.46 ± 20.49	119.54 ± 5.80	310.06 ± 26.37	299.82 ± 61.30	220.22 ± 33.93
$PMGS(Rot)_E$ (S_E)	8.73 ± 2.03	1.48 ± 0.36	20.98 ± 3.86	41.83 ± 8.78	31.05 ± 3.72	2.13 ± 0.99	5.43 ± 0.91	5.86 ± 2.03
ROX	6.05 ± 0.99	3.29 ± 0.62	1.71 ± 0.59	9.50 ± 1.85	6.46 ± 1.52	8.81 ± 0.98	5.82 ± 1.08	10.07 ± 1.53

Table 3.1. *Raw oxygen consumption rates in H9c2 cells treated with SA-22.* High-resolution respirometric assessment of H9c2 cells subjected to HR insult with or without SA-22 (1 μM) treatment. Following permeabilization with digitonin (4 μM), cells were analyzed with the SUIT-008 protocol. OCRs from each respiratory state were obtained by normalizing oxygen flux following each substrate titration to ROX; Basal, Pyruvate (5 μM) and Malate (2 μM) (PM_L), ADP (10 μM) (PM_P), Cytochrome c (10 μM) (PMc_P), Glutamate (10 μM) (PMG_P), Succinate (10 μM) ($PMGS_P$), FCCP (0.3 μM) ($PMGS_E$), Rotenone (0.5 μM) ($PMGS(Rot)_E$), and Antimycin A (2.5 μM) (ROX). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM). Values represent mean ± SEM (n = 4-18 per group). SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. FCCP; Carbonyl cyanide p-trifluoromethoxyphenylhydrazone. HR; hypoxia-reoxygenation. ROX; Residual Oxygen Consumption. P; Pyruvate. $_P$; ADP-stimulated respiration. M; Malate. G; Glutamate. S; Succinate. $_L$; LEAK. $_E$; Maximum uncoupled respiration. N; N-Pathway (i.e. Complex I). OCR; oxygen consumption rate. SUIT; Substrate-uncoupler-inhibitor titration

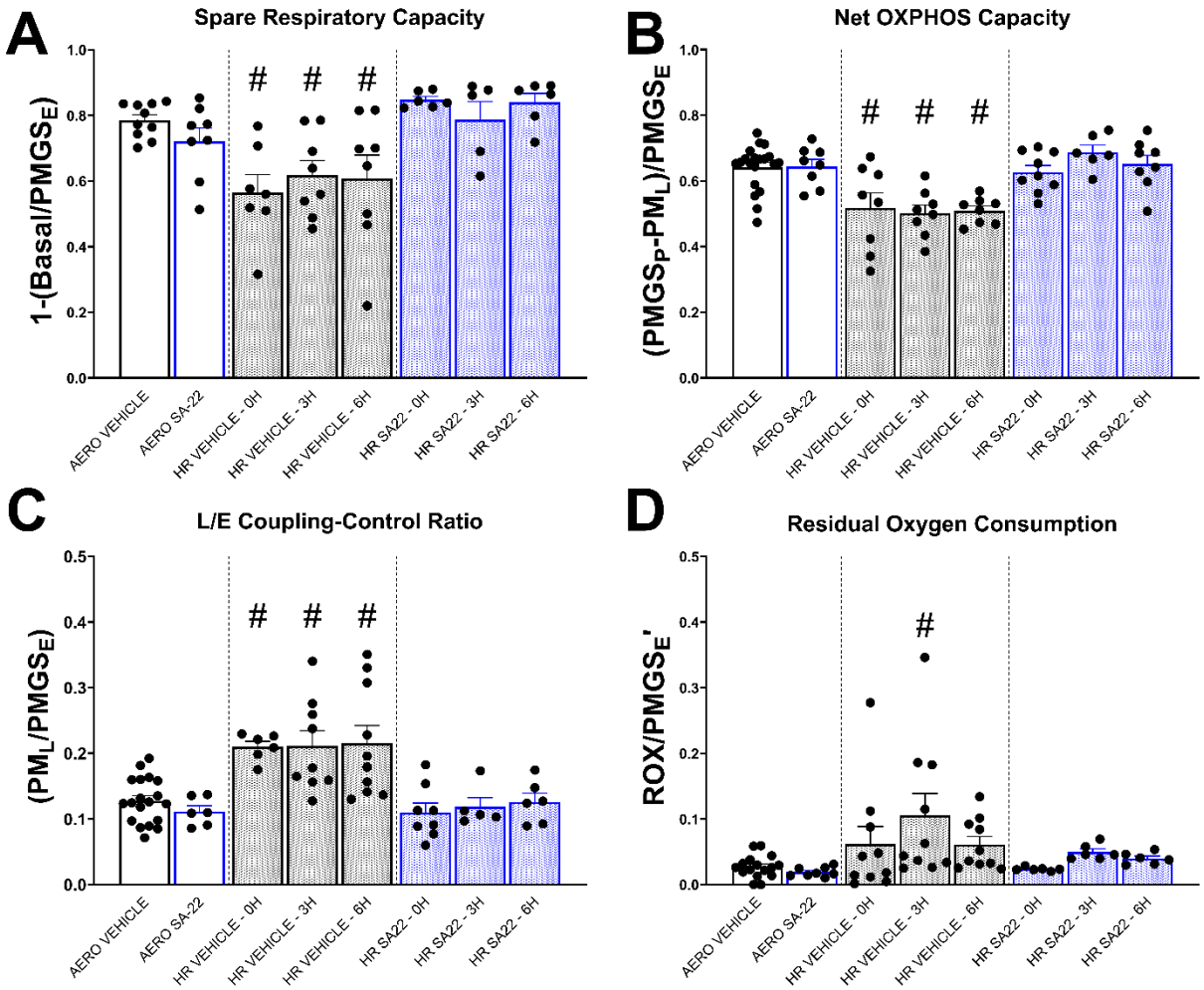

A
Spare Respiratory Capacity
1-(Basal/PMGSE)
B
Net OXPHOS Capacity
(PMGSP-PML)/PMGSE
C
L/E Coupling-Control Ratio
(PML/PMGSE)
D
Residual Oxygen Consumption
ROX/PMGSE'
AERO VEHICLE
AERO SA-22
HR VEHICLE - 0H
HR VEHICLE - 3H
HR VEHICLE - 6H
HR SA22 - 0H
HR SA22 - 3H
HR SA22 - 6H

Figure 3.16. *SA-22 preserves SRC In Vitro.* High-resolution respirometry-based analysis (SUIT-008) of H9c2 cells following HR challenge in the presence or absence of SA-22. Resulting oxygen consumption ratios include SRC (A), net OXPHOS capacity (B), LEAK/ET CCR (C), and ROX (D). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM). Values represent mean ± SEM, # p<0.05 vs aerobic vehicle (n = 5-18 per group). SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. HR; hypoxia-reoxygenation. ROX; Residual Oxygen Consumption. P; Pyruvate. M; Malate. G; Glutamate. S; Succinate. $_{L}$; LEAK. $_{E}$; Maximum uncoupled respiration. $_{P}$; ADP-stimulated respiration. OCR; oxygen consumption rate. SRC; Spare respiratory capacity. CCR; Coupling-control ratio. OXPHOS; Oxidative phosphorylation. SUIT; Substrate-uncoupler-inhibitor titration.

SA-22 preserved complex I respiration stimulated by ADP when compared to HR vehicle **(Figure 3.17A)**. However, this discrepancy appears to largely be a result of cytochrome c loss due to problems with mitochondrial membrane integrity as saturating complex I with glutamate following cytochrome c addition virtually erases any difference between vehicle and SA-22 treated groups by 6-hours post-reoxygenation **(Figure 3.17B, C)**. Interestingly, complex I respiration is significantly reduced by HR only immediately at reoxygenation **(Figure 3.17C)**. SA-22 significantly protects complex I+II respiration compared to HR vehicle groups **(Figure 3.17D)**. Paradoxically, while HR vehicle groups displayed significantly higher complex I-mediated control over NS-pathway respiration prior to uncoupling, rotenone completely inhibited oxygen consumption in aerobic groups and SA-22-treated hypoxic cells after uncoupling **(Figure 3.18A-B)**.

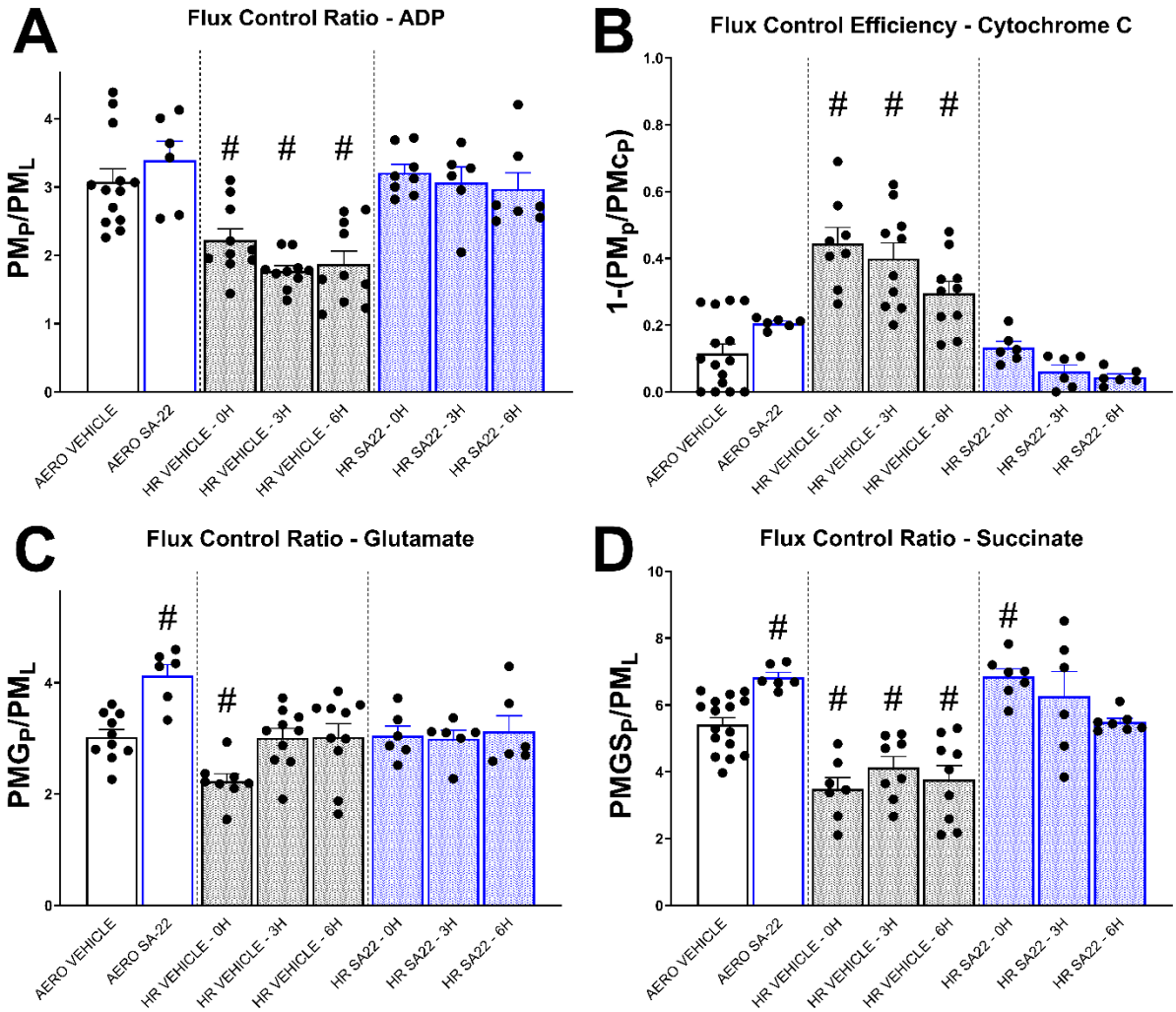

A
Flux Control Ratio - ADP
PMP/PML
B
Flux Control Efficiency - Cytochrome C
1-(PMp/PMCP)
C
Flux Control Ratio - Glutamate
PMGP/PML
D
Flux Control Ratio - Succinate
PMGSP/PML
AERO VEHICLE
AERO SA-22
HR VEHICLE - 0H
HR VEHICLE - 3H
HR VEHICLE - 6H
HR SA22 - 0H
HR SA22 - 3H
HR SA22 - 6H

Figure 3.17. *SA-22 protects complex I and II coupling efficiency against HR challenge.* High-resolution respirometry-based analysis (SUIT-008) of H9c2 cells following HR challenge in the presence or absence of SA-22. Resulting oxygen consumption ratios include PM_P (ADP-stimulated complex I respiration) FCR (A), ADP FCE (B), Cytochrome c FCE (C), PMG_P (saturated complex I respiration) FCR (D), $PMGS_P$ (complex I+II) FCR (E). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM). Values represent mean ± SEM, # p<0.05 vs aerobic vehicle (n = 6-16 per group). SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. HR; hypoxia-reoxygenation. P; Pyruvate. M; Malate. G; Glutamate. S; Succinate. $_L$; LEAK. $_P$; ADP-stimulated respiration. CCR; Coupling-control ratio. FCR; Flux control ratio. FCE; Flux control efficiency. ADP; Adenosine diphosphate. SUIT; Substrate-uncoupler-inhibitor titration.

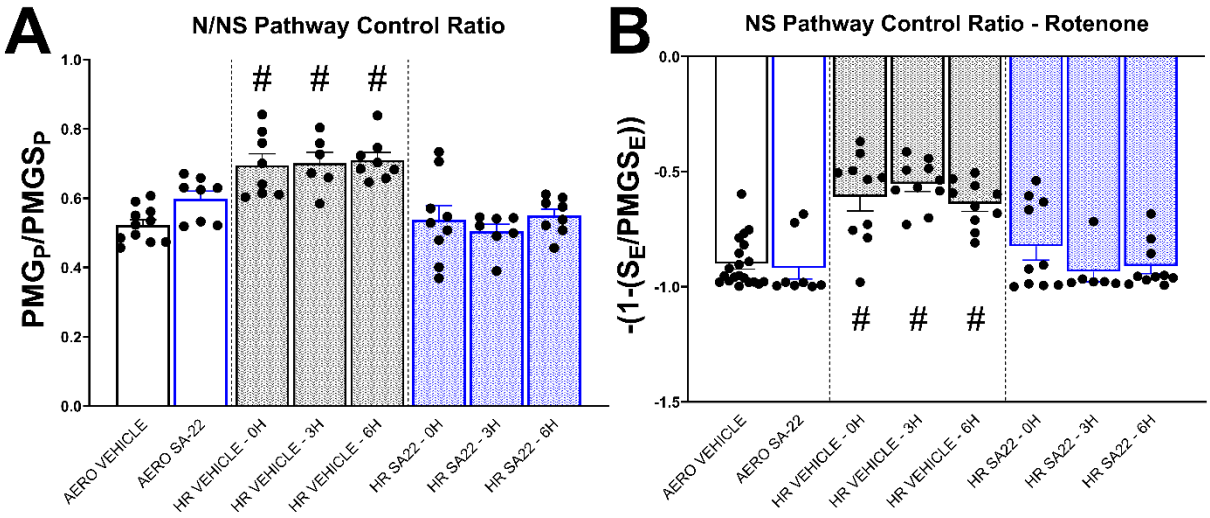


Figure 3.18. *SA-22 treatment preserves Q-junction additivity.* High-resolution respirometry-based analysis (SUIT-008) of H9c2 cells following HR challenge in the presence or absence of SA-22. Resulting oxygen consumption ratios include N/NS PCR (A), and NS PCR(Rot) (B). Treatments; vehicle (0.1% v/v DMSO), SA-22 (1 μM). Values represent mean ± SEM, # p<0.05 vs aerobic vehicle (n = 6-18 per group). SA-22; (10Z,16Z)-18-(3-Ethyloxiran-2-yl)-octadeca-10,16-dienoic acid. HR; hypoxia-reoxygenation. P; Pyruvate. M; Malate. G; Glutamate. S; Succinate/S-Pathway (complex II). $_E$; Maximum uncoupled respiration. $_P$; ADP-stimulated respiration. SUIT; Substrate-uncoupler-inhibitor titration. N; N-Pathway (Complex I). PCR; Pathway control ratio.

CHAPTER 4. DISCUSSION/CONCLUSION

4.1 Summary of results

This thesis presents evidence for the cardioprotective action of a novel synthetic 19,20-EDP analog, SA-22. Preliminary work established that SA-22, similarly to 19,20-EDP, boosts SIRT3 activity directly in the absence of any cellular context **(Figure 1)**. Isolated heart perfusions featuring SA-22 exhibited preserved contractile function dependent on sirtuin activity **(Figure 2)**. Trends in ex vivo tissue function were closely mirrored by ETC complex activity within their mitochondrial fractions **(Figure 3)**. Preservation of ETC function could potentially be correlated with the observation that SA-22 treatment also ameliorated the proteolytic cleavage of OPA1 **(Figure 4C)**. Importantly, we demonstrated that SA-22 maintained cellular SIRT3 and -1 activity, which was reflected in the acetylation state of $MnSOD_2$ and mitochondrial lysine residues **(Figure 5, 6)**. Subsequently, SA-22 abrogated activation of pyroptosis following reperfusion in our murine model of IR injury **(Figure 7)**. Our findings from the *ex vivo* model were then replicated using *in vitro* models of hypoxia-reoxygenation injury featuring cardiac cells. SA-22 preserved cell viability, SIRT3 activity, and inhibited activation of pyroptosis following reoxygenation in H9c2 cells **(Figure 9)**. We again replicated these results using another *in vitro* model of HR injury featuring NRCMs, wherein SA-22 also protected cell viability and prevented activation of pyroptosis **(Figure 10)**. Given that mitochondrial quality control and function are inextricably linked, we sought to explore the potential involvement of SA-22 in mitophagy; Interestingly SA-22 did not appear to evoke changes in the mitochondrial localization of several mitophagy markers in the absence of cellular injury with the exception being parkin **(Figure 11)**. Despite this, both 19,20-EDP and SA-22 clearly exhibited a restorative effect on mitophagy following reoxygenation, dependent

on SIRT3 activity **(Figure 12-14)**. We then sought to bolster our understanding of the effects that SA-22 exerts on mitochondrial respiration. After analyzing H9c2 cells using high-resolution respirometry, we found that not only did SA-22 protect overall mitochondrial respiratory capacity, but that this effect involved preventing deficiencies in ETC complex function and cytochrome c caused by loss of mitochondrial membrane integrity. **(Table 2, Figure 15-16)**. Furthermore, it appears that the protective effects of SA-22 may involve effects on Q-junction additivity following reoxygenation injury **(Figure 17)**.

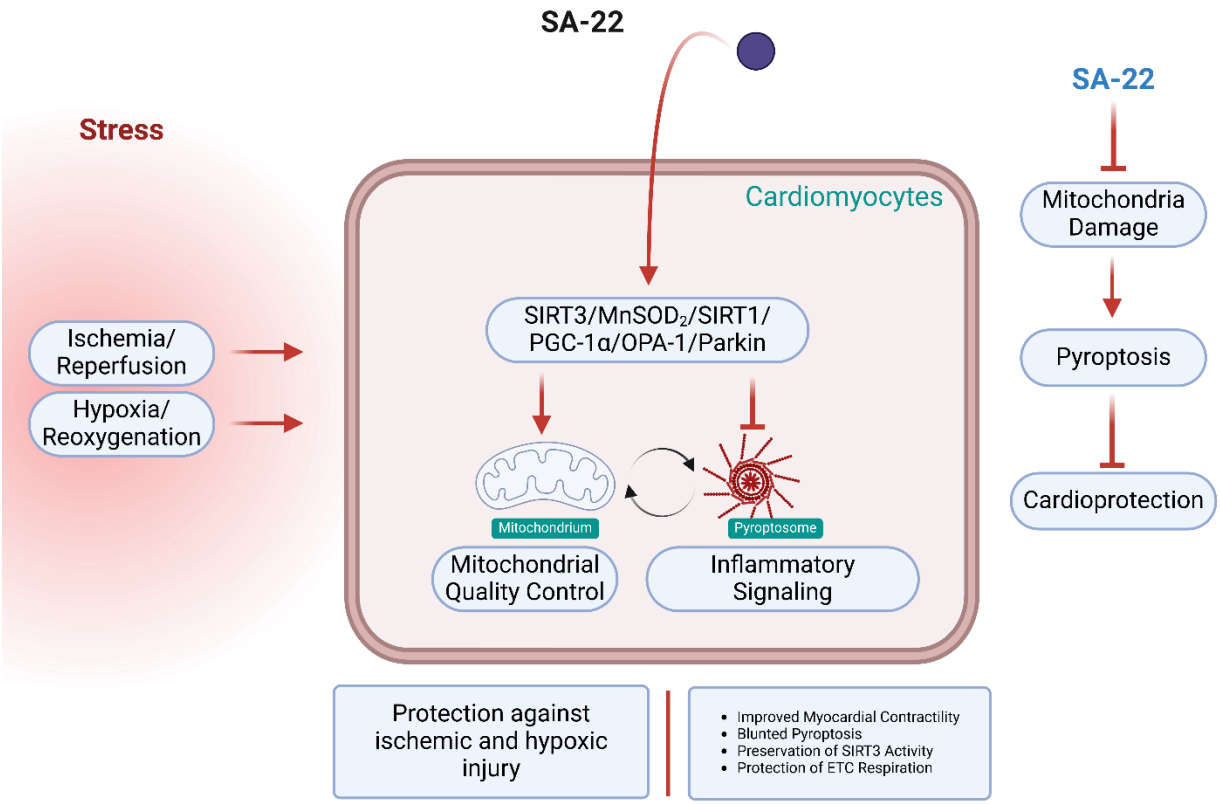


Figure 4.1. *Graphical results summary.*

4.2 Discussion

4.2.1 Application of EDPs as therapeutics

One of the largest hindrances to the translation of therapeutic interventions from experimental models to clinical applications is the unpredictability of MI onset(10). While possessing strong efficacy, therapeutic approaches that focus on ischemic pre-conditioning have failed to translate into practical clinical approaches(411). Furthermore, traditional pharmacological approaches have thus far been unable to lessen the clinical burden and mortality posed by myocardial infarction(10). In recent years, great effort has gone into developing novel delivery systems for the use of biologics to treat myocardial infarction with mixed results(412). Fortunately, in this study we have demonstrated a novel pharmacological agent that remains efficacious even when administered post-ischemically.

While we presume that bioactivity of EDPs specifically within the cardiomyocyte mitochondrium is vital to their cardioprotective properties in the context of ischemic heart disease, mounting evidence indicates that EpFAs are active in several constituents of the myocardium, including endothelial and smooth muscle cells across a plethora of injury models(173,222,278,413). It is imperative to investigate the role of other cell types in EpFA-mediated cardioprotection. However, severe limitations regarding the bioavailability of endogenous EpFAs have hindered their therapeutic utilization until now(277,414,415). In addition to rapid metabolic conversion into diol compounds by soluble epoxide hydrolase, a disadvantage of PUFAs is their strong tight binding interactions with plasma proteins(416,417). Despite this problem however, eicosanoids seem to readily transfer to cells(173,239-241,243,264,278). Theoretically, these

compounds could be administered alongside existing reperfusion protocols (e.g., thrombolytic agents) upon hospitalization. Further elucidation of EDP pharmacokinetics involving off-target interactions and their relationship to experimental efficacy is necessary for the future consideration of EDPs as therapeutic agents. SA-22 is a first-generation mimetic, intended solely for proof-of-concept studies. Subsequent generations of analogs will feature modifications that obviate metabolism by other eicosanoid pathways (e.g., COX and LOX) and reduce auto-oxidation. Additional work characterizing the efficacy of EDPs at various concentrations and how this is affected by chemical modification will be critical for discovering potential candidates for clinical trials. While all our experiments here were conducted using 1 μM epoxide, a concentration informed by previous studies, significant bioactivity of EpFA at nanomolar concentrations has been reported(418). Furthermore, exploration of higher concentrations may yield greater benefits than what has been observed previously.

4.2.2 Sirtuins as potential targets for cardioprotection

Analysis of our data suggests that SA-22 attenuates postischemic injury by ensuring SIRT3 remains active despite insult, which subsequently preserves mitochondrial function and blunts activation of pyroptosis. Sirtuins are a family of NAD^+-dependent deacylase enzymes, consisting of seven members, which can regulate a vast array of proteins via deacetylation activity, including many involved in respiration, oxidative stress responses, metabolism, and mitochondrial dynamics(311,328,329).

The relative pH and abundance of acetyl-CoA contributes to more than 65% of mitochondrial proteins being acetylated under normal physiological conditions. Acetylation of mitochondrial metabolic enzymes (often caused by SIRT3 deficiency) has

been demonstrated to reduce catalytic activity, exacerbate metabolic dysregulation and cause mtROS production(419-421). The mitochondrial antioxidant ROS-scavenger $MnSOD_2$ has its activity significantly upregulated upon deacetylation by SIRT3(332,333). Complexes I and II of the ETC have been established as direct targets for binding and subsequent deacetylation by SIRT3(422). In agreement with the broader literature, this study demonstrated hearts perfused with SA-22, a potential sirtuin-potentiating compound, improved respiratory chain function and lowered mitochondrial protein acetylation **(Figure 3.3, 3.5)**. A rate-limiting factor for sirtuin activity is NAD^+; as such, decreased SIRT3 activity following IR injury could be caused by a reduction in the NAD^+/NADH ratio. Previously, we demonstrated hearts perfused with a synthetic 16,17-EDP analog possessed a much higher NAD^+/NADH ratio than hearts treated with vehicle(240). While NAD^+ deficiency may be in part due to disruption of complex I following reperfusion, it has also been demonstrated that inhibition of the NAD^+-dependent DNA repair enzyme poly-ADP-ribose polymerase 1 (PARP1) following myocardial ischemia in pigs was cardioprotective(423,424). Crucially, as complex I is responsible for regenerating NAD^+ from NADH, preserving its function during the early phases of reperfusion/reoxygenation injury may be important to sustaining SIRT3 activity despite PARP1 hyperactivity (425).

It should be noted that there are limitations to analyzing the effect of SA-22 and SIRT3 activity on each discrete ETC complex separately. Beyond the additive effect of convergent complex I and II flux into the Q-junction, it is well-known that the individual complexes of the respiratory chain RC frequently assemble into super-complexes often dubbed ‘respirasomes’(151-153). The broader literature indicates that respirasome

assembly is marked by significant increases in respiratory efficiency(155-157). Conversely, when respirasome assembly is inhibited or disrupted, marked increases in ROS production can be observed(158-160). Interestingly, increases in ETC complex acetylation induced by SIRT3 KO in cardiac cells have been correlated with respirasome disassembly(161). Therefore, it could be theorized that the beneficial effect of SA-22 treatment with respect to mitochondrial respiration may be more attributable to the protection of respirasome assembly than it is to the effect of direct complex deacetylation.

In addition, SA-22 administration reduced the proteolytic cleavage of OPA1, a protein critically important for proper cristae organization, mitochondrial function, and metabolic adaptation, in response to IR injury(352,426,427). Following the processing of the mature peptide into non-cleaved long and short isoforms, OPA1 localizes to the mitochondrial inner membrane wherein its presence is critical for proper stabilization of ATP synthase(428,429). Importantly, accumulation of short OPA1 isoforms has been demonstrated to promote pathological mitochondrial fission(430). Following mitochondrial depolarization during IR injury, OPA1 dissociates from ATP synthase and is subsequently proteolytically cleaved, exacerbating mitochondrial injury(431). Notably, various groups have suggested that SIRT3 regulates the activity of OPA1 through several different mechanisms. One group demonstrated that via deacetylation, SIRT3 promoted the pro-fusion GTPase activity of OPA1(330). Another group found that through deacetylation and inactivation of nuclear encoded mitochondrial escape 1-like 1 (YME1L1), a mitochondrial metalloproteinase that proteolytically processes OPA1, SIRT3 could promote OXPHOS(352). In an even more indirect mechanism, SIRT3 may influence OPA1 cleavage through deacetylation of ERK and its subsequent effects on mitochondrial

dynamics(432). As OPA1 homeostasis is well-known to be tightly coupled to respiratory function, potentiating SIRT3 activity may help to curtail IR-induced dysregulations in mitochondrial structure and metabolism(433-435).

While we did not explore the involvement of SIRT1 in-depth here, it has long been associated with bioenergetic outcomes following hypoxia/ischemia(55,307,436-438). The most well-characterized function of SIRT1 is the promotion of mitobiogenesis through the deacetylation of PGC-1α(439,440). A previous work by our group observed that 19,20-EDP relied on SIRT1 activity for its cardioprotective effects in a murine *in vitro* model of endotoxemia(222). In this study, we found that SIRT1 activity was also preserved despite IR injury in isolated perfused mouse hearts **(Figure 3.6A)**. However, further study is necessary to determine if this is critically important for the effects of SA-22 or if it is simply a positive side-effect of SIRT3/Complex I signaling. Interestingly, a recent study using primary rat neuronal cultures as an *in vitro* model of cerebral ischemia found that stimulating SIRT1 activity with resveratrol led to a downregulation in PARP1-mediated cell death(441). Another group working on human pulmonary arteriolar smooth muscle cells found that SIRT1 was required for SIRT3-mediated post-hypoxia mitobiogenetic reprogramming(442). Furthermore, additional literature indicates that SIRT1 signals with a plethora of proteins involved with cellular responses to hypoxia including hypoxia-inducible-factor-1α (HIF-1α), nuclear factor erythroid 2-related factor 2 (NRF2), and phosphoinositide 3-kinase (PI3K) (443-445).

Despite not being as extensively studied as SIRT1 and -3, thought should be given to the potential roles of other sirtuins in myocardial diseases. Importantly, all sirtuin homologs possess a highly conserved NAD^+ binding domain and thus have similar

metabolic triggers for their activity (446). However, not all sirtuins possess deacetylase activity and instead may regulate other post-translational modifications (PTMs) such as malonylation, succinylation, and lactylation (447-449). In contrast to the protective action of SIRT1 and SIRT3, recent research seems to indicate that SIRT2 activity in the context of myocardial diseases can be either beneficial or deleterious depending on injury severity (450-452). SIRT4 competitively binds $MnSOD_2$ and sequesters it from SIRT3, thereby increasing ROS and promoting cardiac hypertrophy(453). Another group demonstrated that inhibition of SIRT4 with MicroRNA-497 prevented the development of cardiac hypertrophy post-TAC surgery *in vivo*(454). Desuccinylation activity by SIRT5 has been shown to have significant impact on murine cardiac metabolic function(455,456). A recent study found that SIRT5 played a key role in liver-myocardial crosstalk following acute ischemia(457). The roles of SIRT6 in the context of myocardial ischemia seem more clearly positive, with several groups demonstrating the cardio-protectivity of its action(458-461). In short, while our findings here seem attractive, further study into the potential deleterious effects of sirtuin-potentiation in other cell-types, tissues, and disease models is necessary for the therapeutic consideration of 19,20-EDP derivatives.

Moving forward it will also be important to consider possible sex differences, specifically as they relate to sirtuins. While our group has not observed significant differences between young male and female mice regarding the efficacy of DHA and 19,20-EDP, we have yet to substantively test synthetic epoxylipids in female mice(221). Furthermore, emerging evidence seems to suggest potential significant sex differences in SIRT expression(462,463). Future exploration of these topics may help to rationalize

prior observations regarding sexually dimorphic susceptibility to ischemic heart disease(464).

4.2.3 SA-22 and programmed cell death

Activation of pyroptosis following reperfusion exacerbates tissue injury and contributes to adverse outcomes. Cleavage of GSDMD by caspase-1 leads to semi-permeabilization of the outer plasma membrane and secretion of cell death promoting factors such as IL-1β(399). These factors actively polarize and recruit neighboring macrophages to the site of injury, perpetuating inflammation, and aggravating cell death(41,43,465). A recent study found that GSDMD permeabilizes both the mitochondrial inner membrane (MIM) and -outer membrane (MOM) prior to the plasma membrane, and that this resulted in cytosolic release of cytochrome c, mtROS and mtDNA, leading to enhanced pyroptotic cell death dependent on cardiolipin(466). Coincidentally, cardiolipin has also been implicated in both OPA1 activity as well as the mitochondrial localization of the BH3 interacting-domain death agonist (tBID) (467-469). Interestingly, SIRT3 has been shown to regulate cardiac cardiolipin biosynthesis, activity, and protein binding(470-473). Furthermore, it has been demonstrated that potentiation of sirtuin activity may be a viable strategy for the attenuation of pyroptotic cell death(474,475). Our results indicated that SA-22 attenuated the activation of pyroptotic mediators such as caspase-1, GSDMD, and IL-1β **(Figure 3.7)**. Intriguingly, despite perfusion with NAM greatly worsening myocardial function recovery, these hearts exhibited protection against pyroptosis comparable to hearts perfused with SA-22 alone **(Figure 3.7)**. These data were further supported when we observed significant loss of cytochrome c from HR injured cells during our respirometry experiments **(Figure 3.17)**.

Further work is necessary to identify and characterize potential pleiotropic mechanisms by which SA-22s preserve heart function and protect cardiomyocytes against IR-induced pyroptosis.

4.2.4 SA-22 and mitochondrial homeostasis

Analysis of our data suggests that EDP compounds may have a restorative effect on mitophagy following HR insult **(Figure 3.13-3.15)**. However, it does not appear that this is due to a direct stimulatory effect on traditional PINK1/Parkin-dependent mitophagy as SA-22 did not cause mitochondrial localization of mitophagy markers nor did it stimulate the recruitment of mitochondria to autophagosomes in the absence of cellular injury **(Figure 3.11)**. The one exception to this was Parkin, which significantly localized to the mitochondria following SA-22 treatment **(Figure 3.11C)**. Beyond simply observing the protective effect of SA-22 with respect to mitophagy, it is somewhat difficult to make inferences regarding the potential MoAs by which SA-22 and SIRT3 act, as the broader literature adamantly suggests that SIRT3 exerts a stimulatory effect on PINK1/Parkin-dependent mitophagy(355,476-478). However, there is growing evidence suggesting that PINK1 and Parkin can regulate mitophagy independent of each other(479,480). Further study is necessary to determine if the effect of SA-22 on Parkin localization has meaningful impact on mitochondrial homeostasis following HR injury. Secondly, while treatment with SA-22 appears to strongly protect mitophagic flux against HR injury according to our mito-Keima data, the corresponding western blot data is somewhat unconvincing **(Figure 3.15)**. Analysis of our high-resolution respirometry data suggests that the cardioprotective benefits of SA-22 may be attributable more to effects on mitochondrial respiration than to regulation of mitophagy. Briefly, SA-22 reduced LEAK

and ROX respiration commonly linked to cellular oxidative stress and improved ETC coupling **(Figure 3.16-3.18)**. One could therefore theorize that the gradual reduction in observed mitophagosome count could have more to do with less mitochondria being dysfunctional and subsequently recruited to autophagosomes as a result of preserved respiration than it does with regulation of mitophagy. Importantly, almost all well-characterized mitophagy pathways including PINK1/Parkin and BNIP/NIX among others heavily rely upon kinase activity to transduce signals(401,481,482). Naturally, deficiencies in ATP production due to respiratory dysfunction will cause the inhibition of kinases crucial to mitophagy. Given the abundance of cytotoxic and pro-pyroptotic DAMPs present within mitochondria, preventing the accumulation and permeabilization of dysfunctional mitochondria following ischemia/hypoxia seems central to resolving reperfusion/reoxygenation injury. Indeed, various strategies aimed at preserving mitochondrial integrity and respiratory functionality against ischemia/reperfusion have been shown to be cardioprotective in recent years. Furthermore, the salutary effects of these strategies with respect to mitochondrial autophagy and the accumulation of dysfunctional mitochondria appear to be dependent on ETC function(393,483,484).

4.3 Conclusion

Within this work we investigated the effects of SA-22, a novel synthetic structurally mimetic analog of the epoxylipid 19,20-EDP, in several experimental models of myocardial ischemic/hypoxic injury. We hypothesized that SA-22 protects cardiomyocytes against IR/HR injury by preserving mitochondrial homeostasis and limiting innate immune responses via potentiation of SIRT3 activity. Using isolated young male C57/Bl6 mouse hearts within an *ex vivo* model of IR injury, we demonstrate that

treatment with SA-22 preserves myocardial contractile function, mitochondrial respiration, and SIRT3 activity, consequently blunting activation of pyroptosis. We supported these findings by replicating them using two *in vitro* models of HR injury, wherein SA-22 also protected cardiac cell viability, mitochondrial respiration, mitophagy, and inhibited pyroptosis. Secondly, our data suggests that SA-22-induced protection is dependent on maintaining sirtuin activity, as pharmacological inhibition of sirtuins abrogated many of the benefits evoked by SA-22 treatment. Although the exact molecular mechanisms of action for SA-22 requires further elucidation, this study provides further evidence for the viability of synthetic EpFA analogs as a therapeutic agent for IR injury.

4.4 Future directions

The present thesis has focused on replication of previous observations that 19,20-EDP exerted its cardioprotective properties dependent on SIRT3, with a novel synthetic analog named SA-22. The secondary fundamental consideration of this work was to establish the functional impact of a direct interaction between SA-22 and SIRT3 as it pertains to mitochondrial homeostasis and pyroptosis following myocardial ischemic injury. In this work, we presented evidence that much like native 19,20-EDP, SA-22 mitigates cardiac IR injury via potentiation of SIRT3 activity, marked by preservation of mitochondrial homeostasis. While we suggest the 19,20-EDP analog SA-22 is cardioprotective in our models of IR and HR injury, another previous work by our group demonstrated cytotoxic effects of 19,20-EDP on H9c2 cells dependent on glycolysis(485). Therefore, it appears that SA-22's biological activity, along with many other EpFAs, is likely dependent on many factors such as time, tissue, metabolism, concentration, sex, age, and species. While this work provided some mechanistic insight into the action of

EDP compound, it is consideration for these other factors that will be critical for the continued study of EpFAs. Thus, the conclusions of our work identify several directions in which future research can hopefully be taken to translate our findings into the development of novel therapeutic agents.

Of primary important is to assess EDP compounds for direct interactions with sirtuins other than SIRT3. Given the vastness of the sirtuin interactome, further study into the functional relationships between EDPs and the other sirtuins should elucidate much of how EDPs regulate mitochondrial homeostasis(486).

Given the broader impetus for this field of study is to develop efficacious therapies for IHD, subsequent projects should focus on developing novel EDP-based pharmacophores that feature more advanced structural modifications (i.e., urea substitutions that obviate sEH metabolism) and assess changes in their stability and bioavailability(276,487). Additionally, extensive understanding of the mechanisms behind a therapeutic agent's bioactivity is important for the adoption of their use into clinical practice. Thus, future studies should determine if EDPs are the ligands of unknown receptors that influence their biological activity.

REFERENCES

1. Lopez, A. D., and Adair, T. (2019) Is the long-term decline in cardiovascular-disease mortality in high-income countries over? Evidence from national vital statistics net. *International Journal of Epidemiology* **48**, 1815-1823
2. Timmis, A., Vardas, P., Townsend, N., Torbica, A., Katus, H., De Smedt, D., Gale, C. P., Maggioni, A. P., Petersen, S. E., Huculeci, R., Kazakiewicz, D., de Benito Rubio, V., Ignatiuk, B., Raisi-Estabragh, Z., Pawlak, A., Karagiannidis, E., Treskes, R., Gaita, D., Beltrame, J. F., McConnachie, A., Bardinet, I., Graham, I., Flather, M., Elliott, P., Mossialos, E. A., Weidinger, F., and Achenbach, S. (2022) European Society of Cardiology: cardiovascular disease statistics 2021. *European heart journal* **43**, 716-799
3. Mancini, G. B., Gosselin, G., Chow, B., Kostuk, W., Stone, J., Yvorchuk, K. J., Abramson, B. L., Cartier, R., Huckell, V., Tardif, J. C., Connelly, K., Ducas, J., Farkouh, M. E., Gupta, M., Juneau, M., O'Neill, B., Raggi, P., Teo, K., Verma, S., Zimmermann, R., and Canadian Cardiovascular, S. (2014) Canadian Cardiovascular Society guidelines for the diagnosis and management of stable ischemic heart disease. *Can J Cardiol* **30**, 837-849
4. Canada, P. H. A. o. (2018) Report from the Canadian Chronic Disease Surveillance System: heart disease in Canada, 2018.
5. Smolderen, K. G., Bell, A., Lei, Y., Cohen, E. A., Steg, P. G., Bhatt, D. L., Mahoney, E. M., and investigators, R. r. (2010) One-year costs associated with cardiovascular disease in Canada: Insights from the REduction of Atherothrombosis for Continued Health (REACH) registry. *Can J Cardiol* **26**, 297-305
6. Populations, B. o. t. H. o. S., and Criteria, C. o. S. S. C. D. (2010) *Cardiovascular disability: updating the Social Security listings*, National Academies Press
7. Grotel, D. M. (1945) The role of coronary atherosclerosis in the functional disturbance of the coronary blood circulation, in the pathogenesis of the obturatio acuta of the coronal arteries and acute infarctis of the myocardium. *Klin Med (Mosk)* **23**, 3-13
8. Brown, J. C., Gerhardt, T. E., and Kwon, E. (2020) Risk factors for coronary artery disease.
9. Ibanez, B., James, S., Agewall, S., Antunes, M. J., Bucciarelli-Ducci, C., Bueno, H., Caforio, A. L. P., Crea, F., Goudevenos, J. A., Halvorsen, S., Hindricks, G., Kastrati, A., Lenzen, M. J., Prescott, E., Roffi, M., Valgimigli, M., Varenhorst, C., Vranckx, P., Widimský, P., Baumbach, A., Bugiardini, R., Coman, I. M., Delgado, V., Fitzsimons, D., Gaemperli, O., Gershlick, A. H., Gielen, S., Harjola, V. P., Katus, H. A., Knuuti, J., Kolh, P., Leclercq, C., Lip, G. Y. H., Morais, J., Neskovic, A. N., Neumann, F. J., Niessner, A., Piepoli, M. F., Richter, D. J., Shlyakhto, E., Simpson, I. A., Steg, P. G., Terkelsen, C. J., Thygesen, K., Windecker, S., Zamorano, J. L., Zeymer, U., Chettibi, M., Hayrapetyan, H. G., Metzler, B., Ibrahimov, F., Sujayeva, V., Beauloye, C., Dizdarevic-Hudic, L., Karamfiloff, K., Skoric, B., Antoniades, L., Tousek, P., Shaheen, S. M., Marandi, T., Niemel€a, M., Kedev, S., Gilard, M., Aladashvili, A., Elsaesser, A., Kanakakis, I. G., Merkely, B., Gudnason, T., Iakobishvili, Z.,

Bolognese, L., Berkinbayev, S., Bajraktari, G., Beishenkulov, M., Zake, I., Lamin, H. B., Gustiene, O., Pereira, B., Xuereb, R. G., Ztot, S., Juliebø, V., Legutko, J., Timoteo, A. T., Tatu-Chit¸oiu, G., Yakovlev, A., Bertelli, L., Nedeljkovic, M., Studencan, M., Bunc, M., de Castro, A. M. G., Petursson, P., Jeger, R., Mourali, M. S., Yildirir, A., Parkhomenko, A., and Gale, C. P. (2018) 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. in *European Heart Journal*, Oxford University Press

10. Bolli, R., and Tang, X. L. (2022) New insights into cardioprotection, gained by adopting the CAESAR standards of rigor. *Basic Res Cardiol* **117**, 57
11. Neumann, F. J., Sousa-Uva, M., Ahlsson, A., Alfonso, F., Banning, A. P., Benedetto, U., Byrne, R. A., Collet, J. P., Falk, V., Head, S. J., Jüni, P., Kastrati, A., Koller, A., Kristensen, S. D., Niebauer, J., Richter, D. J., Seferovic, P. M., Sibbing, D., Stefanini, G. G., Windecker, S., Yadav, R., Zembala, M. O., Wijns, W., Glineur, D., Aboyans, V., Achenbach, S., Agewall, S., Andreotti, F., Barbato, E., Baumbach, A., Brophy, J., Bueno, H., Calvert, P. A., Capodanno, D., Davierwala, P. M., Delgado, V., Dudek, D., Freemantle, N., Funck-Brentano, C., Gaemperli, O., Gielen, S., Gilard, M., Gorenek, B., Haasenritter, J., Haude, M., Ibanez, B., Iung, B., Jeppsson, A., Katritsis, D., Knuuti, J., Kolh, P., Leite-Moreira, A., Lund, L. H., Maisano, F., Mehilli, J., Metzler, B., Montalescot, G., Pagano, D., Petronio, A. S., Piepoli, M. F., Popescu, B. A., Sádaba, R., Shlyakhto, E., Silber, S., Simpson, I. A., Sparv, D., Tavilla, G., Thiele, H., Tousek, P., Van Belle, E., Vranckx, P., Witkowski, A., Zamorano, J. L., and Roffi, M. (2019) 2018 ESC/EACTS Guidelines on myocardial revascularization. in *European Heart Journal*, Oxford University Press
12. Kuznetsov, A. V., Javadov, S., Margreiter, R., Grimm, M., Hagenbuchner, J., and Ausserlechner, M. J. (2019) The role of mitochondria in the mechanisms of cardiac ischemia-reperfusion injury. in *Antioxidants*, MDPI
13. Soares, R. O. S., Losada, D. M., Jordani, M. C., Évora, P., and Castro-E-Silva, O. (2019) Ischemia/reperfusion injury revisited: An overview of the latest pharmacological strategies. in *International Journal of Molecular Sciences*, MDPI AG
14. Murry, C. E., Reinecke, H., and Pabon, L. M. (2006) Regeneration Gaps. Observations on Stem Cells and Cardiac Repair. in *Journal of the American College of Cardiology*
15. Tzahor, E., and Poss, K. D. (2017) Cardiac regeneration strategies: Staying young at heart. in *Science*, American Association for the Advancement of Science
16. C. Bompotis, G., Deftereos, S., Angelidis, C., Choidis, E., Panagopoulou, V., Kaoukis, A., P. Vassilikos, V., W. Cleman, M., and Giannopoulos, G. (2016) Altered Calcium Handling in Reperfusion Injury. *Medicinal Chemistry* **12**, 114-130
17. Pittas, K., Vrachatis, D. A., Angelidis, C., Tsoucala, S., Giannopoulos, G., and Deftereos, S. (2019) The Role of Calcium Handling Mechanisms in Reperfusion Injury. *Current Pharmaceutical Design* **24**, 4077-4089
18. Xu, T., Ding, W., Ao, X., Chu, X., Wan, Q., Wang, Y., Xiao, D., Yu, W., Li, M., Yu, F., and Wang, J. (2019) ARC regulates programmed necrosis and myocardial

ischemia/reperfusion injury through the inhibition of mPTP opening. *Redox Biology* **20**, 414-426
19. Halestrap, A. P., Doran, E., Gillespie, J. P., and O'Toole, A. (2000) Mitochondria and cell death. *Biochem Soc Trans* **28**, 170-177
20. Maroko, P. R., Kjekshus, J. K., Sobel, B. E., Watanabe, T., Covell, J. W., Ross, J., Jr., and Braunwald, E. (1971) Factors influencing infarct size following experimental coronary artery occlusions. *Circulation* **43**, 67-82
21. Bolli, R., Becker, L., Gross, G., Mentzer, R., Jr., Balshaw, D., Lathrop, D. A., and Ischemia, N. W. G. o. t. T. o. T. f. P. t. H. f. (2004) Myocardial protection at a crossroads: the need for translation into clinical therapy. *Circ Res* **95**, 125-134
22. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**, 1124-1136
23. Heusch, G. (2020) Myocardial ischaemia-reperfusion injury and cardioprotection in perspective. *Nat Rev Cardiol* **17**, 773-789
24. Hausenloy, D. J., Candilio, L., Evans, R., Ariti, C., Jenkins, D. P., Kolvekar, S., Knight, R., Kunst, G., Laing, C., Nicholas, J., Pepper, J., Robertson, S., Xenou, M., Clayton, T., Yellon, D. M., and Investigators, E. T. (2015) Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. *N Engl J Med* **373**, 1408-1417
25. Hausenloy, D. J., Kharbanda, R. K., Moller, U. K., Ramlall, M., Aaroe, J., Butler, R., Bulluck, H., Clayton, T., Dana, A., Dodd, M., Engstrom, T., Evans, R., Lassen, J. F., Christensen, E. F., Garcia-Ruiz, J. M., Gorog, D. A., Hjort, J., Houghton, R. F., Ibanez, B., Knight, R., Lippert, F. K., Lonborg, J. T., Maeng, M., Milasinovic, D., More, R., Nicholas, J. M., Jensen, L. O., Perkins, A., Radovanovic, N., Rakhit, R. D., Ravkilde, J., Ryding, A. D., Schmidt, M. R., Riddervold, I. S., Sorensen, H. T., Stankovic, G., Varma, M., Webb, I., Terkelsen, C. J., Greenwood, J. P., Yellon, D. M., Botker, H. E., and Investigators, C.-E.-P. (2019) Effect of remote ischaemic conditioning on clinical outcomes in patients with acute myocardial infarction (CONDI-2/ERIC-PPCI): a single-blind randomised controlled trial. *Lancet* **394**, 1415-1424
26. Meybohm, P., Bein, B., Brosteanu, O., Cremer, J., Gruenewald, M., Stoppe, C., Coburn, M., Schaelte, G., Boning, A., Niemann, B., Roesner, J., Kletzin, F., Strouhal, U., Reyher, C., Laufenberg-Feldmann, R., Ferner, M., Brandes, I. F., Bauer, M., Stehr, S. N., Kortgen, A., Wittmann, M., Baumgarten, G., Meyer-Treschan, T., Kienbaum, P., Heringlake, M., Schon, J., Sander, M., Treskatsch, S., Smul, T., Wolwender, E., Schilling, T., Fuernau, G., Hasenclever, D., Zacharowski, K., and Collaborators, R. I. S. (2015) A Multicenter Trial of Remote Ischemic Preconditioning for Heart Surgery. *N Engl J Med* **373**, 1397-1407
27. Martins De Oliveira, J., and Levy, M. N. (1960) Effects of hyaluronidase upon the water content of ischemic myocardium. *Am Heart J* **60**, 106-109
28. Day, T. D. (1952) The permeability of interstitial connective tissue and the nature of the interfibrillary substance. *J Physiol* **117**, 1-8
29. Tilton, R. G., Cole, P. A., Larson, K. B., Kilo, C., and Williamson, J. R. (1985) Hyaluronidase does not prevent deterioration of vascular functional integrity during reperfusion after no-flow ischemia in isolated rabbit hearts. *Circ Res* **56**, 839-850

30. (1986) Hyaluronidase therapy for acute myocardial infarction: results of a randomized, blinded, multicenter trial. MILIS Study Group. *Am J Cardiol* **57**, 1236-1243
31. van den Heuvel, A. F., van Gilst, W. H., van Veldhuisen, D. J., de Vries, R. J., Dunselman, P. H., and Kingma, J. H. (1997) Long-term anti-ischemic effects of angiotensin-converting enzyme inhibition in patients after myocardial infarction. The Captopril and Thrombolysis Study (CATS) Investigators. *J Am Coll Cardiol* **30**, 400-405
32. Schaer, G. L., Hursey, T. L., Abrahams, S. L., Buddemeier, K., Ennis, B., Rodriguez, E. R., Hubbell, J. P., Moy, J., and Parrillo, J. E. (1994) Reduction in reperfusion-induced myocardial necrosis in dogs by RheothRx injection (poloxamer 188 N.F.), a hemorheological agent that alters neutrophil function. *Circulation* **90**, 2964-2975
33. O'Keefe, J. H., Grines, C. L., DeWood, M. A., Schaer, G. L., Browne, K., Magorien, R. D., Kalbfleisch, J. M., Fletcher, W. O., Jr., Bateman, T. M., and Gibbons, R. J. (1996) Poloxamer-188 as an adjunct to primary percutaneous transluminal coronary angioplasty for acute myocardial infarction. *Am J Cardiol* **78**, 747-750
34. Puscas, A., Stefanescu, R., Vari, C. E., Osz, B. E., Filip, C., Bitzan, J. K., But, M. G., and Tero-Vescan, A. (2024) Biochemical Aspects That Lead to Abusive Use of Trimetazidine in Performance Athletes: A Mini-Review. *Int J Mol Sci* **25**
35. Mehrotra, T. N., and Bassadone, E. T. (1967) Trimetazidine in the treatment of angina pectoris. *Br J Clin Pract* **21**, 553-554
36. Stanley, W. C., Lopaschuk, G. D., Hall, J. L., and McCormack, J. G. (1997) Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. *Cardiovasc Res* **33**, 243-257
37. (2000) Effect of 48-h intravenous trimetazidine on short- and long-term outcomes of patients with acute myocardial infarction, with and without thrombolytic therapy; A double-blind, placebo-controlled, randomized trial. The EMIP-FR Group. European Myocardial Infarction Project--Free Radicals. *Eur Heart J* **21**, 1537-1546
38. Fenton, R. A., Dickson, E. W., Meyer, T. E., and Dobson, J. G., Jr. (2000) Aging reduces the cardioprotective effect of ischemic preconditioning in the rat heart. *J Mol Cell Cardiol* **32**, 1371-1375
39. Przyklenk, K., Li, G., Simkhovich, B. Z., and Kloner, R. A. (2003) Mechanisms of myocardial ischemic preconditioning are age related: PKC-epsilon does not play a requisite role in old rabbits. *J Appl Physiol (1985)* **95**, 2563-2569
40. Mentzer, R. M., Jr., Birjiniuk, V., Khuri, S., Lowe, J. E., Rahko, P. S., Weisel, R. D., Wellons, H. A., Barker, M. L., and Lasley, R. D. (1999) Adenosine myocardial protection: preliminary results of a phase II clinical trial. *Ann Surg* **229**, 643-649; discussion 649-650
41. Shi, H., Gao, Y., Dong, Z., Yang, J., Gao, R., Li, X., Zhang, S., Ma, L., Sun, X., Wang, Z., Zhang, F., Hu, K., Sun, A., and Ge, J. (2021) GSDMD-Mediated Cardiomyocyte Pyroptosis Promotes Myocardial I/R Injury. *Circ Res* **129**, 383-396
42. Toldo, S., and Abbate, A. (2018) The NLRP3 inflammasome in acute myocardial infarction. *Nat Rev Cardiol* **15**, 203-214

43. Toldo, S., Mauro, A. G., Cutter, Z., and Abbate, A. (2018) Inflammasome, pyroptosis, and cytokines in myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* **315**, H1553-h1568
44. Bergsbaken, T., Fink, S. L., and Cookson, B. T. (2009) Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* **7**, 99-109
45. Fink, S. L., and Cookson, B. T. (2006) Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol* **8**, 1812-1825
46. Sutterwala, F. S., Ogura, Y., Szczepanik, M., Lara-Tejero, M., Lichtenberger, G. S., Grant, E. P., Bertin, J., Coyle, A. J., Galan, J. E., Askenase, P. W., and Flavell, R. A. (2006) Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* **24**, 317-327
47. Proell, M., Riedl, S. J., Fritz, J. H., Rojas, A. M., and Schwarzenbacher, R. (2008) The Nod-like receptor (NLR) family: a tale of similarities and differences. *PLoS One* **3**, e2119
48. Rock, K. L., Latz, E., Ontiveros, F., and Kono, H. (2010) The sterile inflammatory response. *Annu Rev Immunol* **28**, 321-342
49. Mezzaroma, E., Toldo, S., and Abbate, A. (2013) Role of NLRP3 (cryopyrin) in acute myocardial infarction. *Cardiovasc Res* **99**, 225-226
50. Li, H., Yang, D. H., Zhang, Y., Zheng, F., Gao, F., Sun, J., and Shi, G. (2022) Geniposide suppresses NLRP3 inflammasome-mediated pyroptosis via the AMPK signaling pathway to mitigate myocardial ischemia/reperfusion injury. *Chin Med* **17**, 73
51. Ward, R., Li, W., Abdul, Y., Jackson, L., Dong, G., Jamil, S., Filosa, J., Fagan, S. C., and Ergul, A. (2019) NLRP3 inflammasome inhibition with MCC950 improves diabetes-mediated cognitive impairment and vasoneuronal remodeling after ischemia. *Pharmacol Res* **142**, 237-250
52. Wu, X., Wang, B., Zhou, Y., Yang, Z., Jiang, L., Kou, Z., Li, J., Ma, X., and Song, J. (2023) NLRP3 inflammasome inhibitor MCC950 reduces cerebral ischemia/reperfusion induced neuronal ferroptosis. *Neurosci Lett* **795**, 137032
53. Bellut, M., Papp, L., Bieber, M., Kraft, P., Stoll, G., and Schuhmann, M. K. (2021) NLPR3 inflammasome inhibition alleviates hypoxic endothelial cell death in vitro and protects blood-brain barrier integrity in murine stroke. *Cell Death Dis* **13**, 20
54. Chen, M. L., Zhu, X. H., Ran, L., Lang, H. D., Yi, L., and Mi, M. T. (2017) Trimethylamine-N-Oxide Induces Vascular Inflammation by Activating the NLRP3 Inflammasome Through the SIRT3-SOD2-mtROS Signaling Pathway. *J Am Heart Assoc* **6**
55. Kadono, K., Kageyama, S., Nakamura, K., Hirao, H., Ito, T., Kojima, H., Dery, K. J., Li, X., and Kupiec-Weglinski, J. W. (2022) Myeloid Ikaros-SIRT1 signaling axis regulates hepatic inflammation and pyroptosis in ischemia-stressed mouse and human liver. *J Hepatol* **76**, 896-909
56. Xie, Z., Ying, Q., Luo, H., Qin, M., Pang, Y., Hu, H., Zhong, J., Song, Y., Zhang, Z., and Zhang, X. (2023) Resveratrol Alleviates Retinal Ischemia-Reperfusion Injury by

Inhibiting the NLRP3/Gasdermin D/Caspase-1/Interleukin-1beta Pyroptosis Pathway. *Invest Ophthalmol Vis Sci* **64**, 28
57. Luo, C. J., Li, T., Li, H. L., Zhou, Y., and Li, L. (2023) Resveratrol pretreatment alleviates NLRP3 inflammasome-mediated cardiomyocyte pyroptosis by targeting TLR4/MyD88/NF-kappaB signaling cascade in coronary microembolization-induced myocardial damage. *Korean J Physiol Pharmacol* **27**, 143-155
58. Chang, Y. P., Ka, S. M., Hsu, W. H., Chen, A., Chao, L. K., Lin, C. C., Hsieh, C. C., Chen, M. C., Chiu, H. W., Ho, C. L., Chiu, Y. C., Liu, M. L., and Hua, K. F. (2015) Resveratrol inhibits NLRP3 inflammasome activation by preserving mitochondrial integrity and augmenting autophagy. *J Cell Physiol* **230**, 1567-1579
59. Jassem, W., Fuggle, S. V., Rela, M., Koo, D. D., and Heaton, N. D. (2002) The role of mitochondria in ischemia/reperfusion injury. *Transplantation* **73**, 493-499
60. Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., Brohi, K., Itagaki, K., and Hauser, C. J. (2010) Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* **464**, 104-107
61. Nakahira, K., Hisata, S., and Choi, A. M. (2015) The Roles of Mitochondrial Damage-Associated Molecular Patterns in Diseases. *Antioxid Redox Signal* **23**, 1329-1350
62. Nakahira, K., Haspel, J. A., Rathinam, V. A., Lee, S. J., Dolinay, T., Lam, H. C., Englert, J. A., Rabinovitch, M., Cernadas, M., Kim, H. P., Fitzgerald, K. A., Ryter, S. W., and Choi, A. M. (2011) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* **12**, 222-230
63. Sandanger, O., Ranheim, T., Vinge, L. E., Bliksoen, M., Alfsnes, K., Finsen, A. V., Dahl, C. P., Askevold, E. T., Florholmen, G., Christensen, G., Fitzgerald, K. A., Lien, E., Valen, G., Espevik, T., Aukrust, P., and Yndestad, A. (2013) The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia-reperfusion injury. *Cardiovasc Res* **99**, 164-174
64. Biasizzo, M., and Kopitar-Jerala, N. (2020) Interplay Between NLRP3 Inflammasome and Autophagy. *Front Immunol* **11**, 591803
65. Kawane, K., Ohtani, M., Miwa, K., Kizawa, T., Kanbara, Y., Yoshioka, Y., Yoshikawa, H., and Nagata, S. (2006) Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* **443**, 998-1002
66. Oka, T., Hikoso, S., Yamaguchi, O., Taneike, M., Takeda, T., Tamai, T., Oyabu, J., Murakawa, T., Nakayama, H., Nishida, K., Akira, S., Yamamoto, A., Komuro, I., and Otsu, K. (2012) Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature* **485**, 251-255
67. Rongvaux, A., Jackson, R., Harman, C. C., Li, T., West, A. P., de Zoete, M. R., Wu, Y., Yordy, B., Lakhani, S. A., Kuan, C. Y., Taniguchi, T., Shadel, G. S., Chen, Z. J., Iwasaki, A., and Flavell, R. A. (2014) Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. *Cell* **159**, 1563-1577
68. West, A. P., Khoury-Hanold, W., Staron, M., Tal, M. C., Pineda, C. M., Lang, S. M., Bestwick, M., Duguay, B. A., Raimundo, N., MacDuff, D. A., Kaech, S. M., Smiley, J. R., Means, R. E., Iwasaki, A., and Shadel, G. S. (2015) Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* **520**, 553-557

69. White, M. J., McArthur, K., Metcalf, D., Lane, R. M., Cambier, J. C., Herold, M. J., van Delft, M. F., Bedoui, S., Lessene, G., Ritchie, M. E., Huang, D. C., and Kile, B. T. (2014) Apoptotic caspases suppress mtDNA-induced STING-mediated type I IFN production. *Cell* **159**, 1549-1562
70. Davis, B. K., Wen, H., and Ting, J. P. (2011) The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* **29**, 707-735
71. Rathinam, V. A., Vanaja, S. K., and Fitzgerald, K. A. (2012) Regulation of inflammasome signaling. *Nat Immunol* **13**, 333-342
72. Campbell, C. T., Kolesar, J. E., and Kaufman, B. A. (2012) Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. *Biochim Biophys Acta* **1819**, 921-929
73. Kang, D., Kim, S. H., and Hamasaki, N. (2007) Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. *Mitochondrion* **7**, 39-44
74. Crouser, E. D., Shao, G., Julian, M. W., Macre, J. E., Shadel, G. S., Tridandapani, S., Huang, Q., and Wewers, M. D. (2009) Monocyte activation by necrotic cells is promoted by mitochondrial proteins and formyl peptide receptors. *Crit Care Med* **37**, 2000-2009
75. Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., and Clayton, D. A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* **18**, 231-236
76. Chaung, W. W., Wu, R., Ji, Y., Dong, W., and Wang, P. (2012) Mitochondrial transcription factor A is a proinflammatory mediator in hemorrhagic shock. *Int J Mol Med* **30**, 199-203
77. Le, Y., Murphy, P. M., and Wang, J. M. (2002) Formyl-peptide receptors revisited. *Trends Immunol* **23**, 541-548
78. Czapiga, M., Gao, J. L., Kirk, A., and Lekstrom-Himes, J. (2005) Human platelets exhibit chemotaxis using functional N-formyl peptide receptors. *Exp Hematol* **33**, 73-84
79. Carp, H. (1982) Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils. *J Exp Med* **155**, 264-275
80. Panaro, M. A., Acquafredda, A., Sisto, M., Lisi, S., Maffione, A. B., and Mitolo, V. (2006) Biological role of the N-formyl peptide receptors. *Immunopharmacol Immunotoxicol* **28**, 103-127
81. Rabiet, M. J., Huet, E., and Boulay, F. (2005) Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. *Eur J Immunol* **35**, 2486-2495
82. Ackrell, B. A., Kearney, E. B., and Singer, T. P. (1978) Mammalian succinate dehydrogenase. *Methods Enzymol* **53**, 466-483
83. Shaham, O., Slate, N. G., Goldberger, O., Xu, Q., Ramanathan, A., Souza, A. L., Clish, C. B., Sims, K. B., and Mootha, V. K. (2010) A plasma signature of human

mitochondrial disease revealed through metabolic profiling of spent media from cultured muscle cells. *Proc Natl Acad Sci U S A* **107**, 1571-1575
84. Rubic, T., Lametschwandtner, G., Jost, S., Hinteregger, S., Kund, J., Carballido-Perrig, N., Schwarzler, C., Junt, T., Voshol, H., Meingassner, J. G., Mao, X., Werner, G., Rot, A., and Carballido, J. M. (2008) Triggering the succinate receptor GPR91 on dendritic cells enhances immunity. *Nat Immunol* **9**, 1261-1269
85. Tannahill, G. M., Curtis, A. M., Adamik, J., Palsson-McDermott, E. M., McGettrick, A. F., Goel, G., Frezza, C., Bernard, N. J., Kelly, B., Foley, N. H., Zheng, L., Gardet, A., Tong, Z., Jany, S. S., Corr, S. C., Haneklaus, M., Caffrey, B. E., Pierce, K., Walmsley, S., Beasley, F. C., Cummins, E., Nizet, V., Whyte, M., Taylor, C. T., Lin, H., Masters, S. L., Gottlieb, E., Kelly, V. P., Clish, C., Auron, P. E., Xavier, R. J., and O'Neill, L. A. (2013) Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* **496**, 238-242
86. Yang, L., Yu, D., Fan, H. H., Feng, Y., Hu, L., Zhang, W. Y., Zhou, K., and Mo, X. M. (2014) Triggering the succinate receptor GPR91 enhances pressure overload-induced right ventricular hypertrophy. *Int J Clin Exp Pathol* **7**, 5415-5428
87. Chicco, A. J., and Sparagna, G. C. (2007) Role of cardiolipin alterations in mitochondrial dysfunction and disease. *Am J Physiol Cell Physiol* **292**, C33-44
88. Claypool, S. M., and Koehler, C. M. (2012) The complexity of cardiolipin in health and disease. *Trends Biochem Sci* **37**, 32-41
89. Paradies, G., Paradies, V., Ruggiero, F. M., and Petrosillo, G. (2014) Cardiolipin and mitochondrial function in health and disease. *Antioxid Redox Signal* **20**, 1925-1953
90. Ott, M., Zhivotovsky, B., and Orrenius, S. (2007) Role of cardiolipin in cytochrome c release from mitochondria. *Cell Death Differ* **14**, 1243-1247
91. Iyer, S. S., He, Q., Janczy, J. R., Elliott, E. I., Zhong, Z., Olivier, A. K., Sadler, J. J., Knepper-Adrian, V., Han, R., Qiao, L., Eisenbarth, S. C., Nauseef, W. M., Cassel, S. L., and Sutterwala, F. S. (2013) Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* **39**, 311-323
92. Page, E., and McCallister, L. P. (1973) Quantitative electron microscopic description of heart muscle cells. Application to normal, hypertrophied and thyroxin-stimulated hearts. *Am J Cardiol* **31**, 172-181
93. Lopaschuk, G. D., Ussher, J. R., Folmes, C. D., Jaswal, J. S., and Stanley, W. C. (2010) Myocardial fatty acid metabolism in health and disease. *Physiol Rev* **90**, 207-258
94. Murphy, E., Ardehali, H., Balaban, R. S., DiLisa, F., Dorn, G. W., 2nd, Kitsis, R. N., Otsu, K., Ping, P., Rizzuto, R., Sack, M. N., Wallace, D., Youle, R. J., American Heart Association Council on Basic Cardiovascular Sciences, C. o. C. C., Council on Functional, G., and Translational, B. (2016) Mitochondrial Function, Biology, and Role in Disease: A Scientific Statement From the American Heart Association. *Circ Res* **118**, 1960-1991
95. Williams, G. S., Boyman, L., and Lederer, W. J. (2015) Mitochondrial calcium and the regulation of metabolism in the heart. *J Mol Cell Cardiol* **78**, 35-45

96. Niemann, B., Schwarzer, M., and Rohrbach, S. (2018) Heart and Mitochondria: Pathophysiology and Implications for Cardiac Surgeons. *Thorac Cardiovasc Surg* **66**, 11-19
97. Margulis, L. (1975) Symbiotic theory of the origin of eukaryotic organelles; criteria for proof. *Symp Soc Exp Biol*, 21-38
98. Honegger, R. (2000) Simon Schwendener (1829-1919) and the Dual Hypothesis of Lichens. *The Bryologist* **103**, 307-313
99. Archibald, J. M. (2015) Endosymbiosis and Eukaryotic Cell Evolution. *Curr Biol* **25**, R911-921
100. Archibald, J. (2014) *One plus one equals one: symbiosis and the evolution of complex life*, Oxford University Press, USA
101. Martin, W., and Kowallik, K. V. (1999) Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren im Pflanzenreiche'. *European Journal of Phycology* **34**, 287-295
102. Margulis, L. (2008) *Symbiotic planet: a new look at evolution*, Basic books
103. Taylor, F. J. R. (1974) II. IMPLICATIONS AND EXTENSIONS OF THE SERIAL ENDOSYMBIOSIS THEORY OF THE ORIGIN OF EUKARYOTES. *TAXON* **23**, 229-258
104. Schimper, A. F. W. (1883) *Ueber die Entwicklung der Chlorophyllkörner und Farbkörper nachtrag*,
105. Martin, W. F., Garg, S., and Zimorski, V. (2015) Endosymbiotic theories for eukaryote origin. *Philos Trans R Soc Lond B Biol Sci* **370**, 20140330
106. Martin, W., and Muller, M. (1998) The hydrogen hypothesis for the first eukaryote. *Nature* **392**, 37-41
107. Lane, N., and Martin, W. (2010) The energetics of genome complexity. *Nature* **467**, 929-934
108. West, J. B. (2022) The strange history of atmospheric oxygen. *Physiol Rep* **10**, e15214
109. Crockford, P. W., Kunzmann, M., Bekker, A., Hayles, J., Bao, H., Halverson, G. P., Peng, Y., Bui, T. H., Cox, G. M., Gibson, T. M., Wörndle, S., Rainbird, R., Lepland, A., Swanson-Hysell, N. L., Master, S., Sreenivas, B., Kuznetsov, A., Krupenik, V., and Wing, B. A. (2019) Claypool continued: Extending the isotopic record of sedimentary sulfate. *Chemical Geology* **513**, 200-225
110. Crockford, P. W., Bar On, Y. M., Ward, L. M., Milo, R., and Halevy, I. (2023) The geologic history of primary productivity. *Curr Biol* **33**, 4741-4750 e4745
111. Pavlov, A. A., and Kasting, J. F. (2002) Mass-independent fractionation of sulfur isotopes in Archean sediments: strong evidence for an anoxic Archean atmosphere. *Astrobiology* **2**, 27-41
112. Schirrmeister, B. E., de Vos, J. M., Antonelli, A., and Bagheri, H. C. (2013) Evolution of multicellularity coincided with increased diversification of cyanobacteria and the Great Oxidation Event. *Proc Natl Acad Sci U S A* **110**, 1791-1796
113. Craig, J. M., Kumar, S., and Hedges, S. B. (2023) The origin of eukaryotes and rise in complexity were synchronous with the rise in oxygen. *Front Bioinform* **3**, 1233281
114. Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191**, 144-148

115. Altmann, R. (1894) *Die Elementarorganismen und ihre Beziehungen zu den Zellen*, Veit
116. Mitchell, P. (2011) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. 1966. *Biochim Biophys Acta* **1807**, 1507-1538
117. Gnaiger, E. (2020) Mitochondrial pathways and respiratory control: An Introduction to OXPHOS Analysis. 5th ed.
118. Mitchell, P. (1972) Chemiosmotic coupling in energy transduction: a logical development of biochemical knowledge. *Membrane structure and mechanisms of biological energy transduction*, 5-24
119. Mitchell, P. (1991) Foundations of vectorial metabolism and osmochemistry. *Bioscience reports* **11**, 297-346
120. Singer, S. J., and Nicolson, G. L. (1972) The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720-731
121. Galemou Yoga, E., Schiller, J., and Zickermann, V. (2021) Ubiquinone Binding and Reduction by Complex I-Open Questions and Mechanistic Implications. *Front Chem* **9**, 672851
122. Quinlan, C. L., Orr, A. L., Perevoshchikova, I. V., Treberg, J. R., Ackrell, B. A., and Brand, M. D. (2012) Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions. *J Biol Chem* **287**, 27255-27264
123. Lang, B., Burger, G., and Bandlow, W. (1974) Activity of reduced ubiquinone: cytochrome c oxidoreductase with various ubiquinol-isoprenologues as substrate and corresponding inhibitory effect of antimycin in yeast. *Biochim Biophys Acta* **368**, 71-85
124. Papa, S., Lorusso, M., and Guerrieri, F. (1975) Mechanism of respiration-driven proton translocation in the inner mitochondrial membrane. Analysis of proton translocation associated with oxidation of endogenous ubiquinol. *Biochim Biophys Acta* **387**, 425-440
125. Li, Y., Park, J. S., Deng, J. H., and Bai, Y. (2006) Cytochrome c oxidase subunit IV is essential for assembly and respiratory function of the enzyme complex. *J Bioenerg Biomembr* **38**, 283-291
126. Boyer, P. D. (1997) The ATP synthase--a splendid molecular machine. *Annu Rev Biochem* **66**, 717-749
127. Kocherginsky, N. (2009) Acidic lipids, H(+)-ATPases, and mechanism of oxidative phosphorylation. Physico-chemical ideas 30 years after P. Mitchell's Nobel Prize award. *Prog Biophys Mol Biol* **99**, 20-41
128. Deshpande, O. A., and Mohiuddin, S. S. (2024) Biochemistry, Oxidative Phosphorylation. in *StatPearls*, Treasure Island (FL). pp
129. Zhao, R. Z., Jiang, S., Zhang, L., and Yu, Z. B. (2019) Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int J Mol Med* **44**, 3-15
130. Cadenas, E., and Davies, K. J. (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* **29**, 222-230
131. Turrens, J. F. (2003) Mitochondrial formation of reactive oxygen species. *J Physiol* **552**, 335-344

132. Brand, M. D. (2016) Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic Biol Med* **100**, 14-31
133. Hernansanz-Agustin, P., Ramos, E., Navarro, E., Parada, E., Sanchez-Lopez, N., Pelaez-Aguado, L., Cabrera-Garcia, J. D., Tello, D., Buendia, I., Marina, A., Egea, J., Lopez, M. G., Bogdanova, A., and Martinez-Ruiz, A. (2017) Mitochondrial complex I deactivation is related to superoxide production in acute hypoxia. *Redox Biol* **12**, 1040-1051
134. Quinlan, C. L., Gerencser, A. A., Treberg, J. R., and Brand, M. D. (2011) The mechanism of superoxide production by the antimycin-inhibited mitochondrial Q-cycle. *J Biol Chem* **286**, 31361-31372
135. Muller, F. L., Liu, Y., and Van Remmen, H. (2004) Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem* **279**, 49064-49073
136. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* **237**, 408-414
137. Muramoto, K., Ohta, K., Shinzawa-Itoh, K., Kanda, K., Taniguchi, M., Nabekura, H., Yamashita, E., Tsukihara, T., and Yoshikawa, S. (2010) Bovine cytochrome c oxidase structures enable O2 reduction with minimization of reactive oxygens and provide a proton-pumping gate. *Proc Natl Acad Sci U S A* **107**, 7740-7745
138. Heid, M. E., Keyel, P. A., Kamga, C., Shiva, S., Watkins, S. C., and Salter, R. D. (2013) Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol* **191**, 5230-5238
139. Zhou, R., Tardivel, A., Thorens, B., Choi, I., and Tschopp, J. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* **11**, 136-140
140. Orrenius, S., Gogvadze, V., and Zhivotovsky, B. (2007) Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol* **47**, 143-183
141. Coombes, J. S., Powers, S. K., Demirel, H. A., Jessup, J., Vincent, H. K., Hamilton, K. L., Naito, H., Shanely, R. A., Sen, C. K., Packer, L., and Ji, L. L. (2000) Effect of combined supplementation with vitamin E and alpha-lipoic acid on myocardial performance during in vivo ischaemia-reperfusion. *Acta Physiol Scand* **169**, 261-269
142. Zeltcer, G., Berenshtein, E., Kitrossky, N., Chevion, M., and Samuni, A. (2002) Time window of nitroxide effect on myocardial ischemic-reperfusion injury potentiated by iron. *Free Radic Biol Med* **32**, 912-919
143. Tompkins, A. J., Burwell, L. S., Digerness, S. B., Zaragoza, C., Holman, W. L., and Brookes, P. S. (2006) Mitochondrial dysfunction in cardiac ischemia-reperfusion injury: ROS from complex I, without inhibition. *Biochim Biophys Acta* **1762**, 223-231
144. Cingolani, H. E., Perez, N. G., Aiello, E. A., Ennis, I. L., Garciarena, C. D., Villa-Abrille, M. C., Dulce, R. A., Caldiz, C. I., Yeves, A. M., Correa, M. V., Nolly, M. B., and Chiappe de Cingolani, G. (2008) Early signals after stretch leading to cardiac hypertrophy. Key role of NHE-1. *Front Biosci* **13**, 7096-7114
145. Palomeque, J., Rueda, O. V., Sapia, L., Valverde, C. A., Salas, M., Petroff, M. V., and Mattiazzi, A. (2009) Angiotensin II-induced oxidative stress resets the Ca2+

dependence of Ca2+-calmodulin protein kinase II and promotes a death pathway conserved across different species. *Circ Res* **105**, 1204-1212
146. Hulsmans, M., Van Dooren, E., and Holvoet, P. (2012) Mitochondrial reactive oxygen species and risk of atherosclerosis. *Curr Atheroscler Rep* **14**, 264-276
147. Lorenzo, O., Ramirez, E., Picatoste, B., Egido, J., and Tunon, J. (2013) Alteration of energy substrates and ROS production in diabetic cardiomyopathy. *Mediators Inflamm* **2013**, 461967
148. Chance, B., Estabrook, R. W., and Lee, C. P. (1963) Electron Transport in the Oxysome. *Science* **140**, 379-380
149. Javadov, S., Jang, S., Chapa-Dubocq, X. R., Khuchua, Z., and Camara, A. K. (2021) Mitochondrial respiratory supercomplexes in mammalian cells: structural versus functional role. *J Mol Med (Berl)* **99**, 57-73
150. Schagger, H., and Pfeiffer, K. (2001) The ratio of oxidative phosphorylation complexes I-V in bovine heart mitochondria and the composition of respiratory chain supercomplexes. *J Biol Chem* **276**, 37861-37867
151. Lemieux, H., Blier, P. U., and Gnaiger, E. (2017) Remodeling pathway control of mitochondrial respiratory capacity by temperature in mouse heart: electron flow through the Q-junction in permeabilized fibers. *Sci Rep* **7**, 2840
152. Chance, B., and Williams, G. R. (1955) A method for the localization of sites for oxidative phosphorylation. *Nature* **176**, 250-254
153. Guo, R., Gu, J., Wu, M., and Yang, M. (2016) Amazing structure of respirasome: unveiling the secrets of cell respiration. *Protein Cell* **7**, 854-865
154. Schagger, H., and Pfeiffer, K. (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J* **19**, 1777-1783
155. Acin-Perez, R., Fernandez-Silva, P., Peleato, M. L., Perez-Martos, A., and Enriquez, J. A. (2008) Respiratory active mitochondrial supercomplexes. *Mol Cell* **32**, 529-539
156. Bianchi, C., Genova, M. L., Parenti Castelli, G., and Lenaz, G. (2004) The mitochondrial respiratory chain is partially organized in a supercomplex assembly: kinetic evidence using flux control analysis. *J Biol Chem* **279**, 36562-36569
157. D'Aurelio, M., Gajewski, C. D., Lenaz, G., and Manfredi, G. (2006) Respiratory chain supercomplexes set the threshold for respiration defects in human mtDNA mutant cybrids. *Hum Mol Genet* **15**, 2157-2169
158. Ni, Y., Hagras, M. A., Konstantopoulou, V., Mayr, J. A., Stuchebrukhov, A. A., and Meierhofer, D. (2019) Mutations in NDUFS1 Cause Metabolic Reprogramming and Disruption of the Electron Transfer. *Cells* **8**
159. Gomez, L. A., Monette, J. S., Chavez, J. D., Maier, C. S., and Hagen, T. M. (2009) Supercomplexes of the mitochondrial electron transport chain decline in the aging rat heart. *Arch Biochem Biophys* **490**, 30-35
160. Rosca, M. G., Vazquez, E. J., Kerner, J., Parland, W., Chandler, M. P., Stanley, W., Sabbah, H. N., and Hoppel, C. L. (2008) Cardiac mitochondria in heart failure: decrease in respirasomes and oxidative phosphorylation. *Cardiovasc Res* **80**, 30-39
161. R, M. P.-R., Chapa-Dubocq, X., Guzman-Hernandez, R., Jang, S., C, A. T.-R., Ayala-Pena, S., and Javadov, S. (2019) The Role of Adenine Nucleotide Translocase in the Assembly of Respiratory Supercomplexes in Cardiac Cells. *Cells* **8**

162. Sabbir, M. G., Taylor, C. G., and Zahradka, P. (2021) CAMKK2 regulates mitochondrial function by controlling succinate dehydrogenase expression, post-translational modification, megacomplex assembly, and activity in a cell-type-specific manner. *Cell Commun Signal* **19**, 98
163. Diaz, F., Enriquez, J. A., and Moraes, C. T. (2012) Cells lacking Rieske iron-sulfur protein have a reactive oxygen species-associated decrease in respiratory complexes I and IV. *Mol Cell Biol* **32**, 415-429
164. Yu, H., Guo, P., Xie, X., Wang, Y., and Chen, G. (2017) Ferroptosis, a new form of cell death, and its relationships with tumourous diseases. *J Cell Mol Med* **21**, 648-657
165. Gao, M., Yi, J., Zhu, J., Minikes, A. M., Monian, P., Thompson, C. B., and Jiang, X. (2019) Role of Mitochondria in Ferroptosis. *Mol Cell* **73**, 354-363 e353
166. Winterbourn, C. C. (1995) Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett* **82-83**, 969-974
167. Zhu, H., and Sun, A. (2018) Programmed necrosis in heart disease: Molecular mechanisms and clinical implications. *J Mol Cell Cardiol* **116**, 125-134
168. Baba, Y., Higa, J. K., Shimada, B. K., Horiuchi, K. M., Suhara, T., Kobayashi, M., Woo, J. D., Aoyagi, H., Marh, K. S., Kitaoka, H., and Matsui, T. (2018) Protective effects of the mechanistic target of rapamycin against excess iron and ferroptosis in cardiomyocytes. *Am J Physiol Heart Circ Physiol* **314**, H659-H668
169. Feng, Y., Madungwe, N. B., Imam Aliagan, A. D., Tombo, N., and Bopassa, J. C. (2019) Liproxstatin-1 protects the mouse myocardium against ischemia/reperfusion injury by decreasing VDAC1 levels and restoring GPX4 levels. *Biochem Biophys Res Commun* **520**, 606-611
170. Cheng, V., Rallabandi, R., Gorusupudi, A., Lucas, S., Rognon, G., Bernstein, P. S., Rainier, J. D., and Conboy, J. C. (2022) Influence of very-long-chain polyunsaturated fatty acids on membrane structure and dynamics. *Biophys J* **121**, 2730-2741
171. Jamieson, K. L., Endo, T., Darwesh, A. M., Samokhvalov, V., and Seubert, J. M. (2017) Cytochrome P450-derived eicosanoids and heart function. *Pharmacol Ther* **179**, 47-83
172. Dawczynski, C., Plagge, J., Jahreis, G., Liebisch, G., Horing, M., Seeliger, C., and Ecker, J. (2022) Dietary PUFA Preferably Modify Ethanolamine-Containing Glycerophospholipids of the Human Plasma Lipidome. *Nutrients* **14**
173. Schunck, W. H., Konkel, A., Fischer, R., and Weylandt, K. H. (2018) Therapeutic potential of omega-3 fatty acid-derived epoxyeicosanoids in cardiovascular and inflammatory diseases. *Pharmacol Ther* **183**, 177-204
174. Rodriguez-Leyva, D., Dupasquier, C. M., McCullough, R., and Pierce, G. N. (2010) The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid. *Can J Cardiol* **26**, 489-496
175. Erkkila, A., de Mello, V. D., Riserus, U., and Laaksonen, D. E. (2008) Dietary fatty acids and cardiovascular disease: an epidemiological approach. *Prog Lipid Res* **47**, 172-187
176. Kritchevsky, D. (1998) History of recommendations to the public about dietary fat. *J Nutr* **128**, 449S-452S

177. McLennan, P., Howe, P., Abeywardena, M., Muggli, R., Raederstorff, D., Mano, M., Rayner, T., and Head, R. (1996) The cardiovascular protective role of docosahexaenoic acid. in *European Journal of Pharmacology*
178. Dyerberg, J., and Bang, H. O. (1979) Lipid metabolism, atherogenesis, and haemostasis in Eskimos: the role of the prostaglandin-3 family. *Haemostasis* **8**, 227-233
179. Yokoyama, M., Origasa, H., Matsuzaki, M., Matsuzawa, Y., Saito, Y., Ishikawa, Y., Oikawa, S., Sasaki, J., Hishida, H., Itakura, H., Kita, T., Kitabatake, A., Nakaya, N., Sakata, T., Shimada, K., Shirato, K., and Japan, E. P. A. l. i. s. I. (2007) Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. *Lancet* **369**, 1090-1098
180. Tavazzi, L., Maggioni, A. P., Marchioli, R., Barlera, S., Franzosi, M. G., Latini, R., Lucci, D., Nicolosi, G. L., Porcu, M., Tognoni, G., and Gissi, H. F. I. (2008) Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial. *Lancet* **372**, 1223-1230
181. Keys, A., Anderson, J. T., and Grande, F. (1957) Prediction of serum-cholesterol responses of man to changes in fats in the diet. *Lancet* **273**, 959-966
182. Mensink, R. P., and Katan, M. B. (1992) Effect of dietary fatty acids on serum lipids and lipoproteins. A meta-analysis of 27 trials. *Arterioscler Thromb* **12**, 911-919
183. Mensink, R. P., Zock, P. L., Kester, A. D., and Katan, M. B. (2003) Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am J Clin Nutr* **77**, 1146-1155
184. von Schacky, C. (2006) A review of omega-3 ethyl esters for cardiovascular prevention and treatment of increased blood triglyceride levels. *Vasc Health Risk Manag* **2**, 251-262
185. von Schacky, C. (2007) Omega-3 fatty acids and cardiovascular disease. *Curr Opin Clin Nutr Metab Care* **10**, 129-135
186. Willett, W. C. (2007) The role of dietary n-6 fatty acids in the prevention of cardiovascular disease. *J Cardiovasc Med (Hagerstown)* **8 Suppl 1**, S42-45
187. Harris, W. S. (2007) Omega-3 fatty acids and cardiovascular disease: a case for omega-3 index as a new risk factor. *Pharmacol Res* **55**, 217-223
188. Simopoulos, A. P. (2008) The omega-6/omega-3 fatty acid ratio, genetic variation, and cardiovascular disease. *Asia Pac J Clin Nutr* **17 Suppl 1**, 131-134
189. Ascherio, A., Rimm, E. B., Stampfer, M. J., Giovannucci, E. L., and Willett, W. C. (1995) Dietary intake of marine n-3 fatty acids, fish intake, and the risk of coronary disease among men. *N Engl J Med* **332**, 977-982
190. Kromhout, D., Giltay, E. J., Geleijnse, J. M., and Alpha Omega Trial, G. (2010) n-3 fatty acids and cardiovascular events after myocardial infarction. *N Engl J Med* **363**, 2015-2026
191. Marchioli, R., Barzi, F., Bomba, E., Chieffo, C., Di Gregorio, D., Di Mascio, R., Franzosi, M. G., Geraci, E., Levantesi, G., Maggioni, A. P., Mantini, L., Marfisi, R. M., Mastrogiuseppe, G., Mininni, N., Nicolosi, G. L., Santini, M., Schweiger, C., Tavazzi, L., Tognoni, G., Tucci, C., Valagussa, F., and Investigators, G. I.-P. (2002) Early

protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation* **105**, 1897-1903
192. Fumagalli, M., Moltke, I., Grarup, N., Racimo, F., Bjerregaard, P., Jorgensen, M. E., Korneliussen, T. S., Gerbault, P., Skotte, L., Linneberg, A., Christensen, C., Brandslund, I., Jorgensen, T., Huerta-Sanchez, E., Schmidt, E. B., Pedersen, O., Hansen, T., Albrechtsen, A., and Nielsen, R. (2015) Greenlandic Inuit show genetic signatures of diet and climate adaptation. *Science* **349**, 1343-1347
193. Egert, S., and Stehle, P. (2011) Impact of n-3 fatty acids on endothelial function: results from human interventions studies. *Curr Opin Clin Nutr Metab Care* **14**, 121-131
194. Shukla, S. K., Gupta, S., Ojha, S. K., and Sharma, S. B. (2010) Cardiovascular friendly natural products: a promising approach in the management of CVD. *Nat Prod Res* **24**, 873-898
195. Khawaja, O. A., Gaziano, J. M., and Djousse, L. (2014) N-3 fatty acids for prevention of cardiovascular disease. *Curr Atheroscler Rep* **16**, 450
196. Mozaffarian, D. (2007) JELIS, fish oil, and cardiac events. *Lancet* **369**, 1062-1063
197. Mozaffarian, D., and Wu, J. H. (2011) Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *J Am Coll Cardiol* **58**, 2047-2067
198. Swedberg, K. (2011) n-3 Fatty acids in cardiovascular disease. *N Engl J Med* **365**, 1159; author reply 1159
199. Bieche, I., Narjoz, C., Asselah, T., Vacher, S., Marcellin, P., Lidereau, R., Beaune, P., and de Waziers, I. (2007) Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenet Genomics* **17**, 731-742
200. Nebert, D. W., Wikvall, K., and Miller, W. L. (2013) Human cytochromes P450 in health and disease. *Philos Trans R Soc Lond B Biol Sci* **368**, 20120431
201. Lindros, K. O. (1997) Zonation of cytochrome P450 expression, drug metabolism and toxicity in liver. *Gen Pharmacol* **28**, 191-196
202. Hoebel, B. G., Steyrer, E., and Graier, W. F. (1998) Origin and function of epoxyeicosatrienoic acids in vascular endothelial cells: more than just endothelium-derived hyperpolarizing factor? *Clin Exp Pharmacol Physiol* **25**, 826-830
203. Keeney, D. S., Skinner, C., Wei, S., Friedberg, T., and Waterman, M. R. (1998) A keratinocyte-specific epoxygenase, CYP2B12, metabolizes arachidonic acid with unusual selectivity, producing a single major epoxyeicosatrienoic acid. *J Biol Chem* **273**, 9279-9284
204. Pavek, P., and Dvorak, Z. (2008) Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. *Curr Drug Metab* **9**, 129-143

205. Stegeman, J. J., Woodin, B. R., Klotz, A. V., Wolke, R. E., and Orme-Johnson, N. R. (1982) Cytochrome P-450 and monooxygenase activity in cardiac microsomes from the fish Stenotomus chrysops. *Mol Pharmacol* **21**, 517-526
206. Myasoedova, K. N. (2008) New findings in studies of cytochromes P450. *Biochemistry (Mosc)* **73**, 965-969
207. Gervasini, G., Carrillo, J. A., and Benitez, J. (2004) Potential role of cerebral cytochrome P450 in clinical pharmacokinetics: modulation by endogenous compounds. *Clin Pharmacokinet* **43**, 693-706
208. Wu, S., Chen, W., Murphy, E., Gabel, S., Tomer, K. B., Foley, J., Steenbergen, C., Falck, J. R., Moomaw, C. R., and Zeldin, D. C. (1997) Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. *J Biol Chem* **272**, 12551-12559
209. El-Sherbeni, A. A., and El-Kadi, A. O. (2014) Alterations in cytochrome P450-derived arachidonic acid metabolism during pressure overload-induced cardiac hypertrophy. *Biochem Pharmacol* **87**, 456-466
210. Thum, T., and Borlak, J. (2000) Gene expression in distinct regions of the heart. *Lancet* **355**, 979-983
211. Thum, T., and Borlak, J. (2002) Testosterone, cytochrome P450, and cardiac hypertrophy. *FASEB J* **16**, 1537-1549
212. Zordoky, B. N., Aboutabl, M. E., and El-Kadi, A. O. (2008) Modulation of cytochrome P450 gene expression and arachidonic acid metabolism during isoproterenol-induced cardiac hypertrophy in rats. *Drug Metab Dispos* **36**, 2277-2286
213. VanRollins, M., Baker, R. C., Sprecher, H. W., and Murphy, R. C. (1984) Oxidation of docosahexaenoic acid by rat liver microsomes. *J Biol Chem* **259**, 5776-5783
214. Van Rollins, M., Frade, P. D., and Carretero, O. A. (1988) Oxidation of 5,8,11,14,17-eicosapentaenoic acid by hepatic and renal microsomes. *Biochim Biophys Acta* **966**, 133-149
215. Konkel, A., and Schunck, W. H. (2011) Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids. *Biochim Biophys Acta* **1814**, 210-222
216. Westphal, C., Konkel, A., and Schunck, W. H. (2015) Cytochrome p450 enzymes in the bioactivation of polyunsaturated Fatty acids and their role in cardiovascular disease. *Adv Exp Med Biol* **851**, 151-187
217. Arnold, C., Markovic, M., Blossey, K., Wallukat, G., Fischer, R., Dechend, R., Konkel, A., von Schacky, C., Luft, F. C., Muller, D. N., Rothe, M., and Schunck, W. H. (2010) Arachidonic acid-metabolizing cytochrome P450 enzymes are targets of omega-3 fatty acids. *J Biol Chem* **285**, 32720-32733
218. Fer, M., Dreano, Y., Lucas, D., Corcos, L., Salaun, J. P., Berthou, F., and Amet, Y. (2008) Metabolism of eicosapentaenoic and docosahexaenoic acids by recombinant human cytochromes P450. *Arch Biochem Biophys* **471**, 116-125
219. Barbosa-Sicard, E., Markovic, M., Honeck, H., Christ, B., Muller, D. N., and Schunck, W. H. (2005) Eicosapentaenoic acid metabolism by cytochrome P450 enzymes of the CYP2C subfamily. *Biochem Biophys Res Commun* **329**, 1275-1281
220. Lucas, D., Goulitquer, S., Marienhagen, J., Fer, M., Dreano, Y., Schwaneberg, U., Amet, Y., and Corcos, L. (2010) Stereoselective epoxidation of the last double bond

of polyunsaturated fatty acids by human cytochromes P450. *J Lipid Res* **51**, 1125-1133
221. Darwesh, A. M., Jamieson, K. L., Wang, C., Samokhvalov, V., and Seubert, J. M. (2019) Cardioprotective effects of CYP-derived epoxy metabolites of docosahexaenoic acid involve limiting NLRP3 inflammasome activation. *Canadian Journal of Physiology and Pharmacology* **97**, 544-556
222. Samokhvalov, V., Jamieson, K. L., Vriend, J., Quan, S., and Seubert, J. M. (2015) CYP epoxygenase metabolites of docosahexaenoic acid protect HL-1 cardiac cells against LPS-induced cytotoxicity through SIRT1. *Cell Death Discovery* **1**
223. Yang, S., Lin, L., Chen, J.-X., Lee, C. R., Seubert, J. M., Wang, Y., Wang, H., Chao, Z.-R., Tao, D.-D., Gong, J.-P., Lu, Z.-Y., Wen Wang, D., and Zeldin, D. C. (2007) Cytochrome P450 Epoxygenases Protect Endothelial Cells from Apoptosis Induced by Tumor Necrosis Factor-α via MAPK and PI3K/Akt Signaling Pathways. in *Am J Physiol Heart Circ Physiol*
224. Fang, X., Cai, W., Cheng, Q., Ai, D., Wang, X., Hammock, B. D., Zhu, Y., and Zhang, X. (2018) Omega-3 PUFA attenuate mice myocardial infarction injury by emerging a protective eicosanoid pattern. *Prostaglandins Other Lipid Mediat* **139**, 1-9
225. Ulu, A., Stephen Lee, K. S., Miyabe, C., Yang, J., Hammock, B. G., Dong, H., and Hammock, B. D. (2014) An omega-3 epoxide of docosahexaenoic acid lowers blood pressure in angiotensin-II-dependent hypertension. *J Cardiovasc Pharmacol* **64**, 87-99
226. Hercule, H. C., Salanova, B., Essin, K., Honeck, H., Falck, J. R., Sausbier, M., Ruth, P., Schunck, W. H., Luft, F. C., and Gollasch, M. (2007) The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BK alpha channel subunit in rodents. *Exp Physiol* **92**, 1067-1076
227. Lauterbach, B., Barbosa-Sicard, E., Wang, M. H., Honeck, H., Kargel, E., Theuer, J., Schwartzman, M. L., Haller, H., Luft, F. C., Gollasch, M., and Schunck, W. H. (2002) Cytochrome P450-dependent eicosapentaenoic acid metabolites are novel BK channel activators. *Hypertension* **39**, 609-613
228. Ye, D., Zhang, D., Oltman, C., Dellsperger, K., Lee, H. C., and VanRollins, M. (2002) Cytochrome p-450 epoxygenase metabolites of docosahexaenoate potently dilate coronary arterioles by activating large-conductance calcium-activated potassium channels. *J Pharmacol Exp Ther* **303**, 768-776
229. Zhang, Y., Oltman, C. L., Lu, T., Lee, H. C., Dellsperger, K. C., and VanRollins, M. (2001) EET homologs potently dilate coronary microvessels and activate BK(Ca) channels. *Am J Physiol Heart Circ Physiol* **280**, H2430-2440
230. Lu, T., VanRollins, M., and Lee, H. C. (2002) Stereospecific activation of cardiac ATP-sensitive K(+) channels by epoxyeicosatrienoic acids: a structural determinant study. *Mol Pharmacol* **62**, 1076-1083
231. Lu, T., Ye, D., Wang, X., Seubert, J. M., Graves, J. P., Bradbury, J. A., Zeldin, D. C., and Lee, H. C. (2006) Cardiac and vascular KATP channels in rats are activated by endogenous epoxyeicosatrienoic acids through different mechanisms. *J Physiol* **575**, 627-644

232. Morin, C., Sirois, M., Echave, V., Rizcallah, E., and Rousseau, E. (2009) Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. *Am J Physiol Lung Cell Mol Physiol* **296**, L130-139
233. Schafer, S. M. G., Sendetski, M., Angioni, C., Nusing, R., Geisslinger, G., Scholich, K., and Sisignano, M. (2020) The omega-3 lipid 17,18-EEQ sensitizes TRPV1 and TRPA1 in sensory neurons through the prostacyclin receptor (IP). *Neuropharmacology* **166**, 107952
234. McDougle, D. R., Watson, J. E., Abdeen, A. A., Adili, R., Caputo, M. P., Krapf, J. E., Johnson, R. W., Kilian, K. A., Holinstat, M., and Das, A. (2017) Anti-inflammatory omega-3 endocannabinoid epoxides. *Proc Natl Acad Sci U S A* **114**, E6034-E6043
235. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci U S A* **94**, 4318-4323
236. Ravingerova, T., Carnicka, S., Nemcekova, M., Ledvenyiova, V., Adameova, A., Kelly, T., Barlaka, E., Galatou, E., Khandelwal, V. K., and Lazou, A. (2012) PPAR-alpha activation as a preconditioning-like intervention in rats in vivo confers myocardial protection against acute ischaemia-reperfusion injury: involvement of PI3K-Akt. *Can J Physiol Pharmacol* **90**, 1135-1144
237. Milligan, G., Shimpukade, B., Ulven, T., and Hudson, B. D. (2017) Complex Pharmacology of Free Fatty Acid Receptors. *Chem Rev* **117**, 67-110
238. Wang, S., Awad, K. S., Elinoff, J. M., Dougherty, E. J., Ferreyra, G. A., Wang, J. Y., Cai, R., Sun, J., Ptasinska, A., and Danner, R. L. (2015) G Protein-coupled Receptor 40 (GPR40) and Peroxisome Proliferator-activated Receptor gamma (PPARgamma): AN INTEGRATED TWO-RECEPTOR SIGNALING PATHWAY. *J Biol Chem* **290**, 19544-19557
239. Kranrod, J. W., Darwesh, A. M., Bassiouni, W., Huang, A., Fang, L., Korodimas, J. V., Adebesin, A. M., Munnuri, S., Falck, J. R., and Seubert, J. M. (2024) Cardioprotective Action of a Novel Synthetic 19,20-EDP Analog Is Sirt Dependent. *J Cardiovasc Pharmacol* **83**, 105-115
240. Darwesh, A. M., Bassiouni, W., Adebesin, A. M., Mohammad, A. S., Falck, J. R., and Seubert, J. M. (2020) A Synthetic Epoxydocosapentaenoic Acid Analogue Ameliorates Cardiac Ischemia/Reperfusion Injury: The Involvement of the Sirtuin 3-NLRP3 Pathway. *Int J Mol Sci* **21**
241. Darwesh, A. M., Jamieson, K. L., Wang, C., Samokhvalov, V., and Seubert, J. M. (2019) Cardioprotective effects of CYP-derived epoxy metabolites of docosahexaenoic acid involve limiting NLRP3 inflammasome activation (1). *Can J Physiol Pharmacol* **97**, 544-556
242. Samokhvalov, V., Jamieson, K. L., Vriend, J., Quan, S., and Seubert, J. M. (2015) CYP-epoxygenase metabolites of docosahexaenoic acid protect HL-1 cardiac cells against LPS-induced cytotoxicity Through SIRT1. *Cell Death Discov* **1**, 15054-

243. Samokhvalov, V., Jamieson, K. L., Fedotov, I., Endo, T., and Seubert, J. M. (2016) SIRT Is Required for EDP-Mediated Protective Responses toward Hypoxia-Reoxygenation Injury in Cardiac Cells. *Front Pharmacol* **7**, 124
244. Harris, T. R., and Hammock, B. D. (2013) Soluble epoxide hydrolase: gene structure, expression and deletion. *Gene* **526**, 61-74
245. Morisseau, C., and Hammock, B. D. (2005) Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. *Annu Rev Pharmacol Toxicol* **45**, 311-333
246. Fang, X., Kaduce, T. L., Weintraub, N. L., Harmon, S., Teesch, L. M., Morisseau, C., Thompson, D. A., Hammock, B. D., and Spector, A. A. (2001) Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition. *J Biol Chem* **276**, 14867-14874
247. Weintraub, N. L., Fang, X., Kaduce, T. L., VanRollins, M., Chatterjee, P., and Spector, A. A. (1999) Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids. *Am J Physiol* **277**, H2098-2108
248. Widstrom, R. L., Norris, A. W., Van Der Veer, J., and Spector, A. A. (2003) Fatty acid-binding proteins inhibit hydration of epoxyeicosatrienoic acids by soluble epoxide hydrolase. *Biochemistry* **42**, 11762-11767
249. Carroll, M. A., Balazy, M., Huang, D. D., Rybalova, S., Falck, J. R., and McGiff, J. C. (1997) Cytochrome P450-derived renal HETEs: storage and release. *Kidney Int* **51**, 1696-1702
250. Karara, A., Dishman, E., Falck, J. R., and Capdevila, J. H. (1991) Endogenous epoxyeicosatrienoyl-phospholipids. A novel class of cellular glycerolipids containing epoxidized arachidonate moieties. *J Biol Chem* **266**, 7561-7569
251. Klett, E. L., Chen, S., Yechoor, A., Lih, F. B., and Coleman, R. A. (2017) Long-chain acyl-CoA synthetase isoforms differ in preferences for eicosanoid species and long-chain fatty acids. *J Lipid Res* **58**, 884-894
252. Spector, A. A., Fang, X., Snyder, G. D., and Weintraub, N. L. (2004) Epoxyeicosatrienoic acids (EETs): metabolism and biochemical function. *Prog Lipid Res* **43**, 55-90
253. Zeldin, D. C., Kobayashi, J., Falck, J. R., Winder, B. S., Hammock, B. D., Snapper, J. R., and Capdevila, J. H. (1993) Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *J Biol Chem* **268**, 6402-6407
254. Chaudhary, K. R., Zordoky, B. N., Edin, M. L., Alsaleh, N., El-Kadi, A. O., Zeldin, D. C., and Seubert, J. M. (2013) Differential effects of soluble epoxide hydrolase inhibition and CYP2J2 overexpression on postischemic cardiac function in aged mice. *Prostaglandins Other Lipid Mediat* **104-105**, 8-17
255. Bannehr, M., Lohr, L., Gelep, J., Haverkamp, W., Schunck, W. H., Gollasch, M., and Wutzler, A. (2019) Linoleic Acid Metabolite DiHOME Decreases Post-ischemic Cardiac Recovery in Murine Hearts. *Cardiovasc Toxicol* **19**, 365-371
256. Edin, M. L., Wang, Z., Bradbury, J. A., Graves, J. P., Lih, F. B., DeGraff, L. M., Foley, J. F., Torphy, R., Ronnekleiv, O. K., Tomer, K. B., Lee, C. R., and Zeldin, D. C. (2011) Endothelial expression of human cytochrome P450 epoxygenase CYP2C8 increases

susceptibility to ischemia-reperfusion injury in isolated mouse heart. *FASEB J* **25**, 3436-3447
257. Fleming, I. (2014) The pharmacology of the cytochrome P450 epoxygenase/soluble epoxide hydrolase axis in the vasculature and cardiovascular disease. *Pharmacol Rev* **66**, 1106-1140
258. Zeldin, D. C. (2001) Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem* **276**, 36059-36062
259. Ai, D., Fu, Y., Guo, D., Tanaka, H., Wang, N., Tang, C., Hammock, B. D., Shyy, J. Y., and Zhu, Y. (2007) Angiotensin II up-regulates soluble epoxide hydrolase in vascular endothelium in vitro and in vivo. *Proc Natl Acad Sci U S A* **104**, 9018-9023
260. Ai, D., Pang, W., Li, N., Xu, M., Jones, P. D., Yang, J., Zhang, Y., Chiamvimonvat, N., Shyy, J. Y., Hammock, B. D., and Zhu, Y. (2009) Soluble epoxide hydrolase plays an essential role in angiotensin II-induced cardiac hypertrophy. *Proc Natl Acad Sci U S A* **106**, 564-569
261. Zhao, T. T., Wasti, B., Xu, D. Y., Shen, L., Du, J. Q., and Zhao, S. P. (2012) Soluble epoxide hydrolase and ischemic cardiomyopathy. *Int J Cardiol* **155**, 181-187
262. Wang, Y. X., Ulu, A., Zhang, L. N., and Hammock, B. (2010) Soluble epoxide hydrolase in atherosclerosis. *Curr Atheroscler Rep* **12**, 174-183
263. Ding, Y., Li, Y., Zhang, X., He, J., Lu, D., Fang, X., Wang, Y., Wang, J., Zhang, Y., Qiao, X., Gan, L. M., Chen, C., and Zhu, Y. (2017) Soluble epoxide hydrolase activation by S-nitrosation contributes to cardiac ischemia-reperfusion injury. *J Mol Cell Cardiol* **110**, 70-79
264. Darwesh, A. M., Keshavarz-Bahaghighat, H., Jamieson, K. L., and Seubert, J. M. (2019) Genetic Deletion or Pharmacological Inhibition of Soluble Epoxide Hydrolase Ameliorates Cardiac Ischemia/Reperfusion Injury by Attenuating NLRP3 Inflammasome Activation. *Int J Mol Sci* **20**
265. Huang, P. (2023) Research progress on the protective mechanism of a novel soluble epoxide hydrolase inhibitor TPPU on ischemic stroke. *Front Neurol* **14**, 1083972
266. Leuillier, M., Duflot, T., Menoret, S., Messaoudi, H., Djerada, Z., Groussard, D., Denis, R. G. P., Chevalier, L., Karoui, A., Panthu, B., Thiebaut, P. A., Schmitz-Afonso, I., Nobis, S., Campart, C., Henry, T., Sautreuil, C., Luquet, S. H., Beseme, O., Feliu, C., Peyret, H., Nicol, L., Henry, J. P., Renet, S., Mulder, P., Wan, D., Tesson, L., Heslan, J. M., Duche, A., Jacques, S., Ziegler, F., Brunel, V., Rautureau, G. J. P., Monteil, C., do Rego, J. L., do Rego, J. C., Afonso, C., Hammock, B., Madec, A. M., Pinet, F., Richard, V., Anegon, I., Guignabert, C., Morisseau, C., and Bellien, J. (2023) CRISPR/Cas9-mediated inactivation of the phosphatase activity of soluble epoxide hydrolase prevents obesity and cardiac ischemic injury. *J Adv Res* **43**, 163-174
267. Merkel, M. J., Liu, L., Cao, Z., Packwood, W., Young, J., Alkayed, N. J., and Van Winkle, D. M. (2010) Inhibition of soluble epoxide hydrolase preserves cardiomyocytes: role of STAT3 signaling. *Am J Physiol Heart Circ Physiol* **298**, H679-687
268. Qin, J., Le, Y., Froogh, G., Kandhi, S., Jiang, H., Luo, M., Sun, D., and Huang, A. (2016) Sexually dimorphic adaptation of cardiac function: roles of

epoxyeicosatrienoic acid and peroxisome proliferator-activated receptors. *Physiol Rep* **4**
269. Sosnowski, D. K., Jamieson, K. L., Gruzdev, A., Li, Y., Valencia, R., Yousef, A., Kassiri, Z., Zeldin, D. C., and Seubert, J. M. (2022) Cardiomyocyte-specific disruption of soluble epoxide hydrolase limits inflammation to preserve cardiac function. *Am J Physiol Heart Circ Physiol* **323**, H670-H687
270. Zhao, C., Jiang, X., Peng, L., Zhang, Y., Li, H., Zhang, Q., Wang, Y., Yang, F., Wu, J., Wen, Z., He, Z., Shen, J., Chen, C., and Wang, D. W. (2023) Glimepiride, a novel soluble epoxide hydrolase inhibitor, protects against heart failure via increasing epoxyeicosatrienoic acids. *J Mol Cell Cardiol* **185**, 13-25
271. Kubota, T., Arita, M., Isobe, Y., Iwamoto, R., Goto, T., Yoshioka, T., Urabe, D., Inoue, M., and Arai, H. (2014) Eicosapentaenoic acid is converted via omega-3 epoxygenation to the anti-inflammatory metabolite 12-hydroxy-17,18-epoxyeicosatetraenoic acid. *FASEB J* **28**, 586-593
272. McGiff, J. C., and Quilley, J. (1999) 20-HETE and the kidney: resolution of old problems and new beginnings. *Am J Physiol* **277**, R607-623
273. Oliw, E. H., Okamoto, S., Hornsten, L., and Sato, F. (1992) Biosynthesis of prostaglandins from 17(18)epoxy-eicosatetraenoic acid, a cytochrome P-450 metabolite of eicosapentaenoic acid. *Biochim Biophys Acta* **1126**, 261-268
274. Rand, A. A., Barnych, B., Morisseau, C., Cajka, T., Lee, K. S. S., Panigrahy, D., and Hammock, B. D. (2017) Cyclooxygenase-derived proangiogenic metabolites of epoxyeicosatrienoic acids. *Proc Natl Acad Sci U S A* **114**, 4370-4375
275. Lopez-Vicario, C., Alcaraz-Quiles, J., Garcia-Alonso, V., Rius, B., Hwang, S. H., Titos, E., Lopategi, A., Hammock, B. D., Arroyo, V., and Claria, J. (2015) Inhibition of soluble epoxide hydrolase modulates inflammation and autophagy in obese adipose tissue and liver: role for omega-3 epoxides. *Proc Natl Acad Sci U S A* **112**, 536-541
276. Batchu, S. N., Lee, S. B., Qadhi, R. S., Chaudhary, K. R., El-Sikhry, H., Kodela, R., Falck, J. R., and Seubert, J. M. (2011) Cardioprotective effect of a dual acting epoxyeicosatrienoic acid analogue towards ischaemia reperfusion injury. *Br J Pharmacol* **162**, 897-907
277. Capdevila, J. H., Falck, J. R., and Harris, R. C. (2000) Cytochrome P450 and arachidonic acid bioactivation: molecular and functional properties of the arachidonate monooxygenase. *Journal of Lipid Research* **41**, 163-181
278. Jamieson, K. L., Endo, T., Darwesh, A. M., Samokhvalov, V., and Seubert, J. M. (2017) Cytochrome P450-derived eicosanoids and heart function. in *Pharmacology and Therapeutics*, Elsevier Inc.
279. Imig, J. D., Elmarakby, A., Nithipatikom, K., Wei, S., Capdevila, J. H., Tuniki, V. R., Sangras, B., Anjaiah, S., Manthati, V. L., Sudarshan Reddy, D., and Falck, J. R. (2010) Development of epoxyeicosatrienoic acid analogs with in vivo anti-hypertensive actions. *Front Physiol* **1**, 157
280. Jichova, S., Kopkan, L., Huskova, Z., Dolezelova, S., Neckar, J., Kujal, P., Vernerova, Z., Kramer, H. J., Sadowski, J., Kompanowska-Jezierska, E., Reddy, R. N., Falck, J. R., Imig, J. D., and Cervenka, L. (2016) Epoxyeicosatrienoic acid analog attenuates

the development of malignant hypertension, but does not reverse it once established: a study in Cyp1a1-Ren-2 transgenic rats. *J Hypertens* **34**, 2008-2025
281. Hye Khan, M. A., Pavlov, T. S., Christain, S. V., Neckar, J., Staruschenko, A., Gauthier, K. M., Capdevila, J. H., Falck, J. R., Campbell, W. B., and Imig, J. D. (2014) Epoxyeicosatrienoic acid analogue lowers blood pressure through vasodilation and sodium channel inhibition. *Clin Sci (Lond)* **127**, 463-474
282. Sodhi, K., Puri, N., Inoue, K., Falck, J. R., Schwartzman, M. L., and Abraham, N. G. (2012) EET agonist prevents adiposity and vascular dysfunction in rats fed a high fat diet via a decrease in Bach 1 and an increase in HO-1 levels. *Prostaglandins Other Lipid Mediat* **98**, 133-142
283. Schragenheim, J., Bellner, L., Cao, J., Singh, S. P., Bamshad, D., McClung, J. A., Maayan, O., Meissner, A., Grant, I., Stier, C. T., Jr., and Abraham, N. G. (2018) EET enhances renal function in obese mice resulting in restoration of HO-1-Mfn1/2 signaling, and decrease in hypertension through inhibition of sodium chloride co-transporter. *Prostaglandins Other Lipid Mediat* **137**, 30-39
284. Yeboah, M. M., Hye Khan, M. A., Chesnik, M. A., Sharma, A., Paudyal, M. P., Falck, J. R., and Imig, J. D. (2016) The epoxyeicosatrienoic acid analog PVPA ameliorates cyclosporine-induced hypertension and renal injury in rats. *Am J Physiol Renal Physiol* **311**, F576-585
285. Khan, M. A., Liu, J., Kumar, G., Skapek, S. X., Falck, J. R., and Imig, J. D. (2013) Novel orally active epoxyeicosatrienoic acid (EET) analogs attenuate cisplatin nephrotoxicity. *FASEB J* **27**, 2946-2956
286. Hye Khan, M. A., Fish, B., Wahl, G., Sharma, A., Falck, J. R., Paudyal, M. P., Moulder, J. E., Imig, J. D., and Cohen, E. P. (2016) Epoxyeicosatrienoic acid analogue mitigates kidney injury in a rat model of radiation nephropathy. *Clin Sci (Lond)* **130**, 587-599
287. Hye Khan, M. A., Stavniichuk, A., Sattar, M. A., Falck, J. R., and Imig, J. D. (2019) Epoxyeicosatrienoic Acid Analog EET-A Blunts Development of Lupus Nephritis in Mice. *Front Pharmacol* **10**, 512
288. Hoff, U., Bubalo, G., Fechner, M., Blum, M., Zhu, Y., Pohlmann, A., Hentschel, J., Arakelyan, K., Seeliger, E., Flemming, B., Gurgen, D., Rothe, M., Niendorf, T., Manthati, V. L., Falck, J. R., Haase, M., Schunck, W. H., and Dragun, D. (2019) A synthetic epoxyeicosatrienoic acid analogue prevents the initiation of ischemic acute kidney injury. *Acta Physiol (Oxf)* **227**, e13297
289. Skibba, M., Hye Khan, M. A., Kolb, L. L., Yeboah, M. M., Falck, J. R., Amaradhi, R., and Imig, J. D. (2017) Epoxyeicosatrienoic Acid Analog Decreases Renal Fibrosis by Reducing Epithelial-to-Mesenchymal Transition. *Front Pharmacol* **8**, 406
290. Cao, J., Tsenovoy, P. L., Thompson, E. A., Falck, J. R., Touchon, R., Sodhi, K., Rezzani, R., Shapiro, J. I., and Abraham, N. G. (2015) Agonists of epoxyeicosatrienoic acids reduce infarct size and ameliorate cardiac dysfunction via activation of HO-1 and Wnt1 canonical pathway. *Prostaglandins Other Lipid Mediat* **116-117**, 76-86
291. Neckar, J., Hsu, A., Hye Khan, M. A., Gross, G. J., Nithipatikom, K., Cyprova, M., Benak, D., Hlavackova, M., Sotakova-Kasparova, D., Falck, J. R., Sedmera, D., Kolar,

F., and Imig, J. D. (2018) Infarct size-limiting effect of epoxyeicosatrienoic acid analog EET-B is mediated by hypoxia-inducible factor-1alpha via downregulation of prolyl hydroxylase 3. *Am J Physiol Heart Circ Physiol* **315**, H1148-H1158
292. Achenbach, J., Klingler, F. M., Blocher, R., Moser, D., Hafner, A. K., Rodl, C. B., Kretschmer, S., Kruger, B., Lohr, F., Stark, H., Hofmann, B., Steinhilber, D., and Proschak, E. (2013) Exploring the chemical space of multitarget ligands using aligned self-organizing maps. *ACS Med Chem Lett* **4**, 1169-1172
293. Falck, J. R., Koduru, S. R., Mohapatra, S., Manne, R., Atcha, K. R., Atcha, R., Manthati, V. L., Capdevila, J. H., Christian, S., Imig, J. D., and Campbell, W. B. (2014) 14,15-Epoxyeicosa-5,8,11-trienoic Acid (14,15-EET) surrogates: carboxylate modifications. *J Med Chem* **57**, 6965-6972
294. Hiesinger, K., Wagner, K. M., Hammock, B. D., Proschak, E., and Hwang, S. H. (2019) Development of multitarget agents possessing soluble epoxide hydrolase inhibitory activity. *Prostaglandins Other Lipid Mediat* **140**, 31-39
295. Hye Khan, M. A., Hwang, S. H., Sharma, A., Corbett, J. A., Hammock, B. D., and Imig, J. D. (2016) A dual COX-2/sEH inhibitor improves the metabolic profile and reduces kidney injury in Zucker diabetic fatty rat. *Prostaglandins Other Lipid Mediat* **125**, 40-47
296. Berlin, S., Goette, A., Summo, L., Lossie, J., Gebauer, A., Al-Saady, N., Calo, L., Naccarelli, G., Schunck, W. H., Fischer, R., Camm, A. J., and Dobrev, D. (2020) Assessment of OMT-28, a synthetic analog of omega-3 epoxyeicosanoids, in patients with persistent atrial fibrillation: Rationale and design of the PROMISE-AF phase II study. *Int J Cardiol Heart Vasc* **29**, 100573
297. Adebesin, A. M., Wesser, T., Vijaykumar, J., Konkel, A., Paudyal, M. P., Lossie, J., Zhu, C., Westphal, C., Puli, N., Fischer, R., Schunck, W. H., and Falck, J. R. (2019) Development of Robust 17(R),18(S)-Epoxyeicosatetraenoic Acid (17,18-EEQ) Analogues as Potential Clinical Antiarrhythmic Agents. *J Med Chem* **62**, 10124-10143
298. Shikuma, A., Kami, D., Maeda, R., Suzuki, Y., Sano, A., Taya, T., Ogata, T., Konkel, A., Matoba, S., Schunck, W. H., and Gojo, S. (2022) Amelioration of Endotoxemia by a Synthetic Analog of Omega-3 Epoxyeicosanoids. *Front Immunol* **13**, 825171
299. Kranrod, J., Konkel, A., Valencia, R., Darwesh, A., Fischer, R., Schunck, W.-H., and Seubert, J. M. (2024) Cardioprotective properties of OMT-28, a synthetic analog of omega-3 epoxyeicosanoids. *Journal of Biological Chemistry*, 107372
300. Benzie, I. F. (1996) Lipid peroxidation: a review of causes, consequences, measurement and dietary influences. *Int J Food Sci Nutr* **47**, 233-261
301. Shahidi, F., and Zhong, Y. (2010) Lipid oxidation and improving the oxidative stability. *Chem Soc Rev* **39**, 4067-4079
302. Albert, B. B., Cameron-Smith, D., Hofman, P. L., and Cutfield, W. S. (2013) Oxidation of marine omega-3 supplements and human health. *Biomed Res Int* **2013**, 464921
303. Barnes, P. M., Powell-Griner, E., McFann, K., and Nahin, R. L. (2004) Complementary and alternative medicine use among adults: United States, 2002. *Adv Data*, 1-19

304. Barnes, P. M., Bloom, B., and Nahin, R. L. (2008) Complementary and alternative medicine use among adults and children: United States, 2007. *Natl Health Stat Report*, 1-23
305. Imai, S., and Guarente, L. (2014) NAD+ and sirtuins in aging and disease. *Trends Cell Biol* **24**, 464-471
306. Kauppinen, A., Suuronen, T., Ojala, J., Kaarniranta, K., and Salminen, A. (2013) Antagonistic crosstalk between NF-kappaB and SIRT1 in the regulation of inflammation and metabolic disorders. *Cell Signal* **25**, 1939-1948
307. Chang, H. C., and Guarente, L. (2014) SIRT1 and other sirtuins in metabolism. *Trends Endocrinol Metab* **25**, 138-145
308. Brachmann, C. B., Sherman, J. M., Devine, S. E., Cameron, E. E., Pillus, L., and Boeke, J. D. (1995) The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes Dev* **9**, 2888-2902
309. Kaeberlein, M., McVey, M., and Guarente, L. (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev* **13**, 2570-2580
310. Sauve, A. A., and Youn, D. Y. (2012) Sirtuins: NAD(+)-dependent deacetylase mechanism and regulation. *Curr Opin Chem Biol* **16**, 535-543
311. Nogueiras, R., Habegger, K. M., Chaudhary, N., Finan, B., Banks, A. S., Dietrich, M. O., Horvath, T. L., Sinclair, D. A., Pfluger, P. T., and Tschöp, M. H. (2012) Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. *Physiol Rev* **92**, 1479-1514
312. Alcendor, R. R., Gao, S., Zhai, P., Zablocki, D., Holle, E., Yu, X., Tian, B., Wagner, T., Vatner, S. F., and Sadoshima, J. (2007) Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* **100**, 1512-1521
313. Pillai, V. B., Sundaresan, N. R., Jeevanandam, V., and Gupta, M. P. (2010) Mitochondrial SIRT3 and heart disease. *Cardiovasc Res* **88**, 250-256
314. North, B. J., and Verdin, E. (2007) Interphase nucleo-cytoplasmic shuttling and localization of SIRT2 during mitosis. *PLoS One* **2**, e784
315. Inoue, T., Hiratsuka, M., Osaki, M., Yamada, H., Kishimoto, I., Yamaguchi, S., Nakano, S., Katoh, M., Ito, H., and Oshimura, M. (2007) SIRT2, a tubulin deacetylase, acts to block the entry to chromosome condensation in response to mitotic stress. *Oncogene* **26**, 945-957
316. Kawahara, T. L., Michishita, E., Adler, A. S., Damian, M., Berber, E., Lin, M., McCord, R. A., Ongaigui, K. C., Boxer, L. D., Chang, H. Y., and Chua, K. F. (2009) SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* **136**, 62-74
317. Lombard, D. B. (2009) Sirtuins at the breaking point: SIRT6 in DNA repair. *Aging (Albany NY)* **1**, 12-16
318. Zhong, L., D'Urso, A., Toiber, D., Sebastian, C., Henry, R. E., Vadysirisack, D. D., Guimaraes, A., Marinelli, B., Wikstrom, J. D., Nir, T., Clish, C. B., Vaitheesvaran, B., Iliopoulos, O., Kurland, I., Dor, Y., Weissleder, R., Shirihai, O. S., Ellisen, L. W., Espinosa, J. M., and Mostoslavsky, R. (2010) The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* **140**, 280-293

319. Mostoslavsky, R., Chua, K. F., Lombard, D. B., Pang, W. W., Fischer, M. R., Gellon, L., Liu, P., Mostoslavsky, G., Franco, S., Murphy, M. M., Mills, K. D., Patel, P., Hsu, J. T., Hong, A. L., Ford, E., Cheng, H. L., Kennedy, C., Nunez, N., Bronson, R., Frendewey, D., Auerbach, W., Valenzuela, D., Karow, M., Hottiger, M. O., Hursting, S., Barrett, J. C., Guarente, L., Mulligan, R., Demple, B., Yancopoulos, G. D., and Alt, F. W. (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* **124**, 315-329
320. Ford, E., Voit, R., Liszt, G., Magin, C., Grummt, I., and Guarente, L. (2006) Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes Dev* **20**, 1075-1080
321. Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C., and Horikawa, I. (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell* **16**, 4623-4635
322. Vakhrusheva, O., Smolka, C., Gajawada, P., Kostin, S., Boettger, T., Kubin, T., Braun, T., and Bober, E. (2008) Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. *Circ Res* **102**, 703-710
323. Huang, J. Y., Hirschey, M. D., Shimazu, T., Ho, L., and Verdin, E. (2010) Mitochondrial sirtuins. *Biochim Biophys Acta* **1804**, 1645-1651
324. Sundaresan, N. R., Samant, S. A., Pillai, V. B., Rajamohan, S. B., and Gupta, M. P. (2008) SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. *Mol Cell Biol* **28**, 6384-6401
325. Cooper, H. M., Huang, J. Y., Verdin, E., and Spelbrink, J. N. (2009) A new splice variant of the mouse SIRT3 gene encodes the mitochondrial precursor protein. *PLoS One* **4**, e4986
326. Rose, G., Dato, S., Altomare, K., Bellizzi, D., Garasto, S., Greco, V., Passarino, G., Feraco, E., Mari, V., Barbi, C., BonaFe, M., Franceschi, C., Tan, Q., Boiko, S., Yashin, A. I., and De Benedictis, G. (2003) Variability of the SIRT3 gene, human silent information regulator Sir2 homologue, and survivorship in the elderly. *Exp Gerontol* **38**, 1065-1070
327. Bellizzi, D., Rose, G., Cavalcante, P., Covello, G., Dato, S., De Rango, F., Greco, V., Maggiolini, M., Feraco, E., Mari, V., Franceschi, C., Passarino, G., and De Benedictis, G. (2005) A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. *Genomics* **85**, 258-263
328. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions. *Science* **325**, 834-840
329. Gu, H., Yang, K., Wu, Q., Shen, Z., Li, X., and Sun, C. (2021) A link between protein acetylation and mitochondrial dynamics under energy metabolism: A comprehensive overview. *J Cell Physiol* **236**, 7926-7937

330. Samant, S. A., Zhang, H. J., Hong, Z., Pillai, V. B., Sundaresan, N. R., Wolfgeher, D., Archer, S. L., Chan, D. C., and Gupta, M. P. (2014) SIRT3 deacetylates and activates OPA1 to regulate mitochondrial dynamics during stress. *Mol Cell Biol* **34**, 807-819
331. Dikalova, A. E., Pandey, A., Xiao, L., Arslanbaeva, L., Sidorova, T., Lopez, M. G., Billings, F. T. t., Verdin, E., Auwerx, J., Harrison, D. G., and Dikalov, S. I. (2020) Mitochondrial Deacetylase Sirt3 Reduces Vascular Dysfunction and Hypertension While Sirt3 Depletion in Essential Hypertension Is Linked to Vascular Inflammation and Oxidative Stress. *Circ Res* **126**, 439-452
332. Qiu, X., Brown, K., Hirschey, M. D., Verdin, E., and Chen, D. (2010) Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell Metab* **12**, 662-667
333. Tao, R., Coleman, M. C., Pennington, J. D., Ozden, O., Park, S. H., Jiang, H., Kim, H. S., Flynn, C. R., Hill, S., Hayes McDonald, W., Olivier, A. K., Spitz, D. R., and Gius, D. (2010) Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. *Mol Cell* **40**, 893-904
334. Li, M., Chiu, J. F., Mossman, B. T., and Fukagawa, N. K. (2006) Down-regulation of manganese-superoxide dismutase through phosphorylation of FOXO3a by Akt in explanted vascular smooth muscle cells from old rats. *J Biol Chem* **281**, 40429-40439
335. Tan, W. Q., Wang, K., Lv, D. Y., and Li, P. F. (2008) Foxo3a inhibits cardiomyocyte hypertrophy through transactivating catalase. *J Biol Chem* **283**, 29730-29739
336. Ighodaro, O. M., and Akinloye, O. A. (2018) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine* **54**, 287-293
337. Lopaschuk, G. D., Hansen, C. A., and Neely, J. R. (1986) Fatty acid metabolism in hearts containing elevated levels of CoA. *Am J Physiol* **250**, H351-359
338. Davies, N. J., Lovlin, R. E., and Lopaschuk, G. D. (1992) Effect of exogenous fatty acids on reperfusion arrhythmias in isolated working perfused hearts. *Am J Physiol* **262**, H1796-1801
339. Leong, H. S., Brownsey, R. W., Kulpa, J. E., and Allard, M. F. (2003) Glycolysis and pyruvate oxidation in cardiac hypertrophy--why so unbalanced? *Comp Biochem Physiol A Mol Integr Physiol* **135**, 499-513
340. Reimer, K. A., and Jennings, R. B. (1981) Energy metabolism in the reversible and irreversible phases of severe myocardial ischemia. *Acta Med Scand Suppl* **651**, 19-27
341. Hirschey, M. D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D. B., Grueter, C. A., Harris, C., Biddinger, S., Ilkayeva, O. R., Stevens, R. D., Li, Y., Saha, A. K., Ruderman, N. B., Bain, J. R., Newgard, C. B., Farese, R. V., Jr., Alt, F. W., Kahn, C. R., and Verdin, E. (2010) SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature* **464**, 121-125
342. Sundaresan, N. R., Gupta, M., Kim, G., Rajamohan, S. B., Isbatan, A., and Gupta, M. P. (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* **119**, 2758-2771

343. Ahn, B. H., Kim, H. S., Song, S., Lee, I. H., Liu, J., Vassilopoulos, A., Deng, C. X., and Finkel, T. (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proc Natl Acad Sci U S A* **105**, 14447-14452
344. Schwer, B., Bunkenborg, J., Verdin, R. O., Andersen, J. S., and Verdin, E. (2006) Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc Natl Acad Sci U S A* **103**, 10224-10229
345. Schlicker, C., Gertz, M., Papatheodorou, P., Kachholz, B., Becker, C. F., and Steegborn, C. (2008) Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. *J Mol Biol* **382**, 790-801
346. Cimen, H., Han, M. J., Yang, Y., Tong, Q., Koc, H., and Koc, E. C. (2010) Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria. *Biochemistry* **49**, 304-311
347. Palacios, O. M., Carmona, J. J., Michan, S., Chen, K. Y., Manabe, Y., Ward, J. L., 3rd, Goodyear, L. J., and Tong, Q. (2009) Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. *Aging (Albany NY)* **1**, 771-783
348. Pillai, V. B., Sundaresan, N. R., Kim, G., Gupta, M., Rajamohan, S. B., Pillai, J. B., Samant, S., Ravindra, P. V., Isbatan, A., and Gupta, M. P. (2010) Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway. *J Biol Chem* **285**, 3133-3144
349. Shi, T., Fan, G. Q., and Xiao, S. D. (2010) SIRT3 reduces lipid accumulation via AMPK activation in human hepatic cells. *J Dig Dis* **11**, 55-62
350. Dyck, J. R., and Lopaschuk, G. D. (2006) AMPK alterations in cardiac physiology and pathology: enemy or ally? *J Physiol* **574**, 95-112
351. Ventura-Clapier, R., Garnier, A., and Veksler, V. (2008) Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. *Cardiovasc Res* **79**, 208-217
352. He, J., Shangguan, X., Zhou, W., Cao, Y., Zheng, Q., Tu, J., Hu, G., Liang, Z., Jiang, C., Deng, L., Wang, S., Yang, W., Zuo, Y., Ma, J., Cai, R., Chen, Y., Fan, Q., Dong, B., Xue, W., Tan, H., Qi, Y., Gu, J., Su, B., Eugene Chin, Y., Chen, G., Wang, Q., Wang, T., and Cheng, J. (2021) Glucose limitation activates AMPK coupled SENP1-Sirt3 signalling in mitochondria for T cell memory development. *Nat Commun* **12**, 4371
353. Chan, A. Y., Dolinsky, V. W., Soltys, C. L., Viollet, B., Baksh, S., Light, P. E., and Dyck, J. R. (2008) Resveratrol inhibits cardiac hypertrophy via AMP-activated protein kinase and Akt. *J Biol Chem* **283**, 24194-24201
354. Meng, R. S., Pei, Z. H., Yin, R., Zhang, C. X., Chen, B. L., Zhang, Y., Liu, D., Xu, A. L., and Dong, Y. G. (2009) Adenosine monophosphate-activated protein kinase inhibits cardiac hypertrophy through reactivating peroxisome proliferator-activated receptor-alpha signaling pathway. *Eur J Pharmacol* **620**, 63-70
355. Hu, J., Liu, T., Fu, F., Cui, Z., Lai, Q., Zhang, Y., Yu, B., Liu, F., Kou, J., and Li, F. (2022) Omentin1 ameliorates myocardial ischemia-induced heart failure via SIRT3/FOXO3a-dependent mitochondrial dynamical homeostasis and mitophagy. *J Transl Med* **20**, 447
356. Zhao, J., Wang, G., Han, K., Wang, Y., Wang, L., Gao, J., Zhao, S., Wang, G., Chen, S., Luo, A., Wu, J., and Wang, G. (2022) Mitochondrial PKM2 deacetylation by

procyanidin B2-induced SIRT3 upregulation alleviates lung ischemia/reperfusion injury. *Cell Death Dis* **13**, 594
357. Xiaowei, X., Qian, X., and Dingzhou, Z. (2023) Sirtuin-3 activates the mitochondrial unfolded protein response and reduces cerebral ischemia/reperfusion injury. *Int J Biol Sci* **19**, 4327-4339
358. Wang, Z., Sun, R., Wang, G., Chen, Z., Li, Y., Zhao, Y., Liu, D., Zhao, H., Zhang, F., Yao, J., and Tian, X. (2020) SIRT3-mediated deacetylation of PRDX3 alleviates mitochondrial oxidative damage and apoptosis induced by intestinal ischemia/reperfusion injury. *Redox Biol* **28**, 101343
359. Rui, T., and Tang, Q. (2013) IL-33 attenuates anoxia/reoxygenation-induced cardiomyocyte apoptosis by inhibition of PKCbeta/JNK pathway. *PLoS One* **8**, e56089
360. Xu, M. C., Shi, H. M., Gao, X. F., and Wang, H. (2013) Salidroside attenuates myocardial ischemia-reperfusion injury via PI3K/Akt signaling pathway. *J Asian Nat Prod Res* **15**, 244-252
361. Yamazaki, K., Tanaka, S., Sakata, R., Miwa, S., Oriyanhan, W., Takaba, K., Minakata, K., Marui, A., Ikeda, T., Toyokuni, S., Komeda, M., and Ueda, K. (2013) Protective effect of cardioplegia with poly (ADP-ribose) polymerase-1 inhibitor against myocardial ischemia-reperfusion injury: in vitro study of isolated rat heart model. *J Enzyme Inhib Med Chem* **28**, 143-147
362. Scher, M. B., Vaquero, A., and Reinberg, D. (2007) SirT3 is a nuclear NAD+-dependent histone deacetylase that translocates to the mitochondria upon cellular stress. *Genes Dev* **21**, 920-928
363. Cohen, H. Y., Lavu, S., Bitterman, K. J., Hekking, B., Imahiyerobo, T. A., Miller, C., Frye, R., Ploegh, H., Kessler, B. M., and Sinclair, D. A. (2004) Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* **13**, 627-638
364. Jeong, J., Juhn, K., Lee, H., Kim, S. H., Min, B. H., Lee, K. M., Cho, M. H., Park, G. H., and Lee, K. H. (2007) SIRT1 promotes DNA repair activity and deacetylation of Ku70. *Exp Mol Med* **39**, 8-13
365. Li, S., Banck, M., Mujtaba, S., Zhou, M. M., Sugrue, M. M., and Walsh, M. J. (2010) p53-induced growth arrest is regulated by the mitochondrial SirT3 deacetylase. *PLoS One* **5**, e10486
366. Sauve, A. A., Wolberger, C., Schramm, V. L., and Boeke, J. D. (2006) The biochemistry of sirtuins. *Annu Rev Biochem* **75**, 435-465
367. Cosgrove, M. S., Bever, K., Avalos, J. L., Muhammad, S., Zhang, X., and Wolberger, C. (2006) The structural basis of sirtuin substrate affinity. *Biochemistry* **45**, 7511-7521
368. Jin, L., Wei, W., Jiang, Y., Peng, H., Cai, J., Mao, C., Dai, H., Choy, W., Bemis, J. E., Jirousek, M. R., Milne, J. C., Westphal, C. H., and Perni, R. B. (2009) Crystal structures of human SIRT3 displaying substrate-induced conformational changes. *J Biol Chem* **284**, 24394-24405
369. Min, J., Landry, J., Sternglanz, R., and Xu, R. M. (2001) Crystal structure of a SIR2 homolog-NAD complex. *Cell* **105**, 269-279

370. Hawse, W. F., Hoff, K. G., Fatkins, D. G., Daines, A., Zubkova, O. V., Schramm, V. L., Zheng, W., and Wolberger, C. (2008) Structural insights into intermediate steps in the Sir2 deacetylation reaction. *Structure* **16**, 1368-1377
371. Sauve, A. A., Celic, I., Avalos, J., Deng, H., Boeke, J. D., and Schramm, V. L. (2001) Chemistry of gene silencing: the mechanism of NAD+-dependent deacetylation reactions. *Biochemistry* **40**, 15456-15463
372. Sauve, A. A., and Schramm, V. L. (2003) Sir2 regulation by nicotinamide results from switching between base exchange and deacetylation chemistry. *Biochemistry* **42**, 9249-9256
373. Williams, A. (1976) Dilute acid-catalyzed amide hydrolysis: efficiency of the N-protonation mechanism. *Journal of the American Chemical Society* **98**, 5645-5651
374. Sauve, A. A. (2010) Sirtuin chemical mechanisms. *Biochim Biophys Acta* **1804**, 1591-1603
375. Jackson, M. D., Schmidt, M. T., Oppenheimer, N. J., and Denu, J. M. (2003) Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. *J Biol Chem* **278**, 50985-50998
376. Sauve, A. A., Moir, R. D., Schramm, V. L., and Willis, I. M. (2005) Chemical activation of Sir2-dependent silencing by relief of nicotinamide inhibition. *Mol Cell* **17**, 595-601
377. McClure, J. M., Wierman, M. B., Maqani, N., and Smith, J. S. (2012) Isonicotinamide enhances Sir2 protein-mediated silencing and longevity in yeast by raising intracellular NAD+ concentration. *J Biol Chem* **287**, 20957-20966
378. Guan, X., Lin, P., Knoll, E., and Chakrabarti, R. (2014) Mechanism of inhibition of the human sirtuin enzyme SIRT3 by nicotinamide: computational and experimental studies. *PLoS One* **9**, e107729
379. Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., Zhao, W., Thiyagarajan, M., MacGrogan, D., Rodgers, J. T., Puigserver, P., Sadoshima, J., Deng, H., Pedrini, S., Gandy, S., Sauve, A. A., and Pasinetti, G. M. (2006) Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. *J Biol Chem* **281**, 21745-21754
380. Avalos, J. L., Bever, K. M., and Wolberger, C. (2005) Mechanism of sirtuin inhibition by nicotinamide: altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. *Mol Cell* **17**, 855-868
381. Wiktorowska-Owczarek, A., Berezinska, M., and Nowak, J. Z. (2015) PUFAs: Structures, Metabolism and Functions. *Adv Clin Exp Med* **24**, 931-941
382. Grassl, R., Jandl, C., and Bach, T. (2020) Visible Light-Mediated Photochemical Reactions of 2-(2'-Alkenyloxy)cycloalk-2-enones. *J Org Chem* **85**, 11426-11439
383. Zhang, H. Y., Yamakawa, Y., Matsuya, Y., Toyooka, N., Tohda, C., Awale, S., Li, F., Kadota, S., and Tezuka, Y. (2014) Synthesis of long-chain fatty acid derivatives as a novel anti-Alzheimer's agent. *Bioorg Med Chem Lett* **24**, 604-608
384. Darwesh, A. M., El-Azab, M. F., Abo-Gresha, N. M., El-Sayed, N. M., and Moustafa, Y. M. (2018) Cardioprotective Mechanisms of Exenatide in Isoprenaline-induced Myocardial Infarction: Novel Effects on Myocardial α-Estrogen Receptor Expression and IGF-1/IGF-2 System. *J Cardiovasc Pharmacol* **71**, 160-173

385. Seubert, J., Yang, B., Bradbury, J. A., Graves, J., Degraff, L. M., Gabel, S., Gooch, R., Foley, J., Newman, J., Mao, L., Rockman, H. A., Hammock, B. D., Murphy, E., and Zeldin, D. C. (2004) Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K+ channels and p42/p44 MAPK pathway. *Circ Res* **95**, 506-514
386. Seubert, J. M., Sinal, C. J., Graves, J., DeGraff, L. M., Bradbury, J. A., Lee, C. R., Goralski, K., Carey, M. A., Luria, A., Newman, J. W., Hammock, B. D., Falck, J. R., Roberts, H., Rockman, H. A., Murphy, E., and Zeldin, D. C. (2006) Role of soluble epoxide hydrolase in postischemic recovery of heart contractile function. *Circ Res* **99**, 442-450
387. Branco, A. F., Pereira, S. P., Gonzalez, S., Gusev, O., Rizvanov, A. A., and Oliveira, P. J. (2015) Gene Expression Profiling of H9c2 Myoblast Differentiation towards a Cardiac-Like Phenotype. *PLoS One* **10**, e0129303
388. Kovacic, S., Soltys, C. L., Barr, A. J., Shiojima, I., Walsh, K., and Dyck, J. R. (2003) Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart. *J Biol Chem* **278**, 39422-39427
389. Qadhi, R., Alsaleh, N., Samokhvalov, V., El-Sikhry, H., Bellenger, J., and Seubert, J. M. (2013) Differential responses to docosahexaenoic acid in primary and immortalized cardiac cells. *Toxicol Lett* **219**, 288-297
390. Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L., and Angelini, C. (2012) Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat Protoc* **7**, 1235-1246
391. Seubert, J. M., Darmon, A. J., El-Kadi, A. O., D'Souza, S. J., and Bend, J. R. (2002) Apoptosis in murine hepatoma hepa 1c1c7 wild-type, C12, and C4 cells mediated by bilirubin. *Mol Pharmacol* **62**, 257-264
392. Gnaiger, E. (2009) Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. *Int J Biochem Cell Biol* **41**, 1837-1845
393. Lotz, C., Zhang, J., Fang, C., Liem, D., and Ping, P. (2015) Isoflurane protects the myocardium against ischemic injury via the preservation of mitochondrial respiration and its supramolecular organization. *Anesth Analg* **120**, 265-274
394. Bugga, P., Alam, M. J., Kumar, R., Pal, S., Chattopadyay, N., and Banerjee, S. K. (2022) Sirt3 ameliorates mitochondrial dysfunction and oxidative stress through regulating mitochondrial biogenesis and dynamics in cardiomyoblast. *Cell Signal* **94**, 110309
395. Williams, N. C., and O'Neill, L. A. J. (2018) A Role for the Krebs Cycle Intermediate Citrate in Metabolic Reprogramming in Innate Immunity and Inflammation. *Front Immunol* **9**, 141
396. Finkel, T., and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247
397. Mailloux, R. J. (2020) An Update on Mitochondrial Reactive Oxygen Species Production. *Antioxidants (Basel)* **9**
398. Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M.,

Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., Blagosklonny, M. V., Blomgren, K., Borner, C., Boya, P., Brenner, C., Campanella, M., Candi, E., Carmona-Gutierrez, D., Cecconi, F., Chan, F. K., Chandel, N. S., Cheng, E. H., Chipuk, J. E., Cidlowski, J. A., Ciechanover, A., Cohen, G. M., Conrad, M., Cubillos-Ruiz, J. R., Czabotar, P. E., D'Angiolella, V., Dawson, T. M., Dawson, V. L., De Laurenzi, V., De Maria, R., Debatin, K. M., DeBerardinis, R. J., Deshmukh, M., Di Daniele, N., Di Virgilio, F., Dixit, V. M., Dixon, S. J., Duckett, C. S., Dynlacht, B. D., El-Deiry, W. S., Elrod, J. W., Fimia, G. M., Fulda, S., Garcia-Saez, A. J., Garg, A. D., Garrido, C., Gavathiotis, E., Golstein, P., Gottlieb, E., Green, D. R., Greene, L. A., Gronemeyer, H., Gross, A., Hajnoczky, G., Hardwick, J. M., Harris, I. S., Hengartner, M. O., Hetz, C., Ichijo, H., Jaattela, M., Joseph, B., Jost, P. J., Juin, P. P., Kaiser, W. J., Karin, M., Kaufmann, T., Kepp, O., Kimchi, A., Kitsis, R. N., Klionsky, D. J., Knight, R. A., Kumar, S., Lee, S. W., Lemasters, J. J., Levine, B., Linkermann, A., Lipton, S. A., Lockshin, R. A., Lopez-Otin, C., Lowe, S. W., Luedde, T., Lugli, E., MacFarlane, M., Madeo, F., Malewicz, M., Malorni, W., Manic, G., Marine, J. C., Martin, S. J., Martinou, J. C., Medema, J. P., Mehlen, P., Meier, P., Melino, S., Miao, E. A., Molkentin, J. D., Moll, U. M., Munoz-Pinedo, C., Nagata, S., Nunez, G., Oberst, A., Oren, M., Overholtzer, M., Pagano, M., Panaretakis, T., Pasparakis, M., Penninger, J. M., Pereira, D. M., Pervaiz, S., Peter, M. E., Piacentini, M., Pinton, P., Prehn, J. H. M., Puthalakath, H., Rabinovich, G. A., Rehm, M., Rizzuto, R., Rodrigues, C. M. P., Rubinsztein, D. C., Rudel, T., Ryan, K. M., Sayan, E., Scorrano, L., Shao, F., Shi, Y., Silke, J., Simon, H. U., Sistigu, A., Stockwell, B. R., Strasser, A., Szabadkai, G., Tait, S. W. G., Tang, D., Tavernarakis, N., Thorburn, A., Tsujimoto, Y., Turk, B., Vanden Berghe, T., Vandenabeele, P., Vander Heiden, M. G., Villunger, A., Virgin, H. W., Vousden, K. H., Vucic, D., Wagner, E. F., Walczak, H., Wallach, D., Wang, Y., Wells, J. A., Wood, W., Yuan, J., Zakeri, Z., Zhivotovsky, B., Zitvogel, L., Melino, G., and Kroemer, G. (2018) Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* **25**, 486-541

399. Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., and Lieberman, J. (2016) Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153-158
400. Berezhnov, A. V., Soutar, M. P., Fedotova, E. I., Frolova, M. S., Plun-Favreau, H., Zinchenko, V. P., and Abramov, A. Y. (2016) Intracellular pH Modulates Autophagy and Mitophagy. *J Biol Chem* **291**, 8701-8708
401. Lee, Y., Lee, H. Y., Hanna, R. A., and Gustafsson, A. B. (2011) Mitochondrial autophagy by Bnip3 involves Drp1-mediated mitochondrial fission and recruitment of Parkin in cardiac myocytes. *Am J Physiol Heart Circ Physiol* **301**, H1924-1931
402. Liu, L., Sakakibara, K., Chen, Q., and Okamoto, K. (2014) Receptor-mediated mitophagy in yeast and mammalian systems. *Cell Res* **24**, 787-795
403. Yamada, T., Murata, D., Adachi, Y., Itoh, K., Kameoka, S., Igarashi, A., Kato, T., Araki, Y., Huganir, R. L., Dawson, T. M., Yanagawa, T., Okamoto, K., Iijima, M., and Sesaki, H. (2018) Mitochondrial Stasis Reveals p62-Mediated Ubiquitination in Parkin-

Independent Mitophagy and Mitigates Nonalcoholic Fatty Liver Disease. *Cell Metab* **28**, 588-604 e585
404. Clark, I. E., Dodson, M. W., Jiang, C., Cao, J. H., Huh, J. R., Seol, J. H., Yoo, S. J., Hay, B. A., and Guo, M. (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* **441**, 1162-1166
405. Narendra, D., Tanaka, A., Suen, D. F., and Youle, R. J. (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* **183**, 795-803
406. Tang, W., Lin, D., Chen, M., Li, Z., Zhang, W., Hu, W., and Li, F. (2019) PTEN-mediated mitophagy and APE1 overexpression protects against cardiac hypoxia/reoxygenation injury. *In Vitro Cell Dev Biol Anim* **55**, 741-748
407. Yu, J., Nagasu, H., Murakami, T., Hoang, H., Broderick, L., Hoffman, H. M., and Horng, T. (2014) Inflammasome activation leads to Caspase-1-dependent mitochondrial damage and block of mitophagy. *Proc Natl Acad Sci U S A* **111**, 15514-15519
408. Liu, R., Xu, C., Zhang, W., Cao, Y., Ye, J., Li, B., Jia, S., Weng, L., Liu, Y., Liu, L., and Zheng, M. (2022) FUNDC1-mediated mitophagy and HIF1alpha activation drives pulmonary hypertension during hypoxia. *Cell Death Dis* **13**, 634
409. Katayama, H., Kogure, T., Mizushima, N., Yoshimori, T., and Miyawaki, A. (2011) A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem Biol* **18**, 1042-1052
410. Keilin, D. (1929) Cytochrome and Respiratory Enzymes. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character* **104**, 206-252
411. Williams, T. M., Waksman, R., De Silva, K., Jacques, A., and Mahmoudi, M. (2015) Ischemic preconditioning-an unfulfilled promise. *Cardiovasc Revasc Med* **16**, 101-108
412. Borrelli, M. A., Turnquist, H. R., and Little, S. R. (2021) Biologics and their delivery systems: Trends in myocardial infarction. *Adv Drug Deliv Rev* **173**, 181-215
413. Bercea, C. I., Cottrell, G. S., Tamagnini, F., and McNeish, A. J. (2021) Omega-3 polyunsaturated fatty acids and hypertension: a review of vasodilatory mechanisms of docosahexaenoic acid and eicosapentaenoic acid. *Br J Pharmacol* **178**, 860-877
414. Cinelli, M. A., Yang, J., Scharmen, A., Woodman, J., Karchalla, L. M., and Lee, K. S. S. (2018) Enzymatic synthesis and chemical inversion provide both enantiomers of bioactive epoxydocosapentaenoic acids. *J Lipid Res* **59**, 2237-2252
415. Hasegawa, E., Inafuku, S., Mulki, L., Okunuki, Y., Yanai, R., Smith, K. E., Kim, C. B., Klokman, G., Bielenberg, D. R., Puli, N., Falck, J. R., Husain, D., Miller, J. W., Edin, M. L., Zeldin, D. C., Lee, K. S. S., Hammock, B. D., Schunck, W. H., and Connor, K. M. (2017) Cytochrome P450 monooxygenase lipid metabolites are significant second messengers in the resolution of choroidal neovascularization. *Proc Natl Acad Sci U S A* **114**, E7545-E7553
416. Washizaki, K., Smith, Q. R., Rapoport, S. I., and Purdon, A. D. (1994) Brain arachidonic acid incorporation and precursor pool specific activity during

intravenous infusion of unesterified [3H]arachidonate in the anesthetized rat. *J Neurochem* **63**, 727-736
417. Grange, E., Deutsch, J., Smith, Q. R., Chang, M., Rapoport, S. I., and Purdon, A. D. (1995) Specific activity of brain palmitoyl-CoA pool provides rates of incorporation of palmitate in brain phospholipids in awake rats. *J Neurochem* **65**, 2290-2298
418. Falck, J. R., Wallukat, G., Puli, N., Goli, M., Arnold, C., Konkel, A., Rothe, M., Fischer, R., Muller, D. N., and Schunck, W. H. (2011) 17(R),18(S)-epoxyeicosatetraenoic acid, a potent eicosapentaenoic acid (EPA) derived regulator of cardiomyocyte contraction: structure-activity relationships and stable analogues. *J Med Chem* **54**, 4109-4118
419. Hirschey, M. D., Shimazu, T., Jing, E., Grueter, C. A., Collins, A. M., Aouizerat, B., Stancakova, A., Goetzman, E., Lam, M. M., Schwer, B., Stevens, R. D., Muehlbauer, M. J., Kakar, S., Bass, N. M., Kuusisto, J., Laakso, M., Alt, F. W., Newgard, C. B., Farese, R. V., Jr., Kahn, C. R., and Verdin, E. (2011) SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. *Mol Cell* **44**, 177-190
420. Shimazu, T., Hirschey, M. D., Hua, L., Dittenhafer-Reed, K. E., Schwer, B., Lombard, D. B., Li, Y., Bunkenborg, J., Alt, F. W., Denu, J. M., Jacobson, M. P., and Verdin, E. (2010) SIRT3 deacetylates mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 and regulates ketone body production. *Cell Metab* **12**, 654-661
421. Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., Yao, J., Zhou, L., Zeng, Y., Li, H., Li, Y., Shi, J., An, W., Hancock, S. M., He, F., Qin, L., Chin, J., Yang, P., Chen, X., Lei, Q., Xiong, Y., and Guan, K. L. (2010) Regulation of cellular metabolism by protein lysine acetylation. *Science* **327**, 1000-1004
422. Kumar, S., and Lombard, D. B. (2015) Mitochondrial sirtuins and their relationships with metabolic disease and cancer. *Antioxid Redox Signal* **22**, 1060-1077
423. Roesner, J. P., Mersmann, J., Bergt, S., Bohnenberg, K., Barthuber, C., Szabo, C., Noldge-Schomburg, G. E., and Zacharowski, K. (2010) Therapeutic injection of PARP inhibitor INO-1001 preserves cardiac function in porcine myocardial ischemia and reperfusion without reducing infarct size. *Shock* **33**, 507-512
424. Hauser, B., Groger, M., Ehrmann, U., Albicini, M., Bruckner, U. B., Schelzig, H., Venkatesh, B., Li, H., Szabo, C., Speit, G., Radermacher, P., and Kick, J. (2006) The parp-1 inhibitor ino-1001 facilitates hemodynamic stabilization without affecting DNA repair in porcine thoracic aortic cross-clamping-induced ischemia/reperfusion. *Shock* **25**, 633-640
425. Kaniuga, Z. (1963) The Transformation of Mitochondrial Nadh Dehydrogenase into Nadh: Cytochrome C Oxidoreductase. *Biochim Biophys Acta* **73**, 550-564
426. Mishra, P., Carelli, V., Manfredi, G., and Chan, D. C. (2014) Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. *Cell Metab* **19**, 630-641
427. Patten, D. A., Wong, J., Khacho, M., Soubannier, V., Mailloux, R. J., Pilon-Larose, K., MacLaurin, J. G., Park, D. S., McBride, H. M., Trinkle-Mulcahy, L., Harper, M. E., Germain, M., and Slack, R. S. (2014) OPA1-dependent cristae modulation is essential for cellular adaptation to metabolic demand. *EMBO J* **33**, 2676-2691

428. Frezza, C., Cipolat, S., Martins de Brito, O., Micaroni, M., Beznoussenko, G. V., Rudka, T., Bartoli, D., Polishuck, R. S., Danial, N. N., De Strooper, B., and Scorrano, L. (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* **126**, 177-189
429. Song, Z., Chen, H., Fiket, M., Alexander, C., and Chan, D. C. (2007) OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *J Cell Biol* **178**, 749-755
430. Anand, R., Wai, T., Baker, M. J., Kladt, N., Schauss, A. C., Rugarli, E., and Langer, T. (2014) The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. *J Cell Biol* **204**, 919-929
431. Li, X., Li, H., Xu, Z., Ma, C., Wang, T., You, W., Yu, Z., Shen, H., and Chen, G. (2022) Ischemia-induced cleavage of OPA1 at S1 site aggravates mitochondrial fragmentation and reperfusion injury in neurons. *Cell Death Dis* **13**, 321
432. Wang, Q., Xu, J., Li, X., Liu, Z., Han, Y., Xu, X., Li, X., Tang, Y., Liu, Y., Yu, T., and Li, X. (2019) Sirt3 modulate renal ischemia-reperfusion injury through enhancing mitochondrial fusion and activating the ERK-OPA1 signaling pathway. *J Cell Physiol* **234**, 23495-23506
433. Sun, S., Erchova, I., Sengpiel, F., and Votruba, M. (2020) Opa1 Deficiency Leads to Diminished Mitochondrial Bioenergetics With Compensatory Increased Mitochondrial Motility. *Invest Ophthalmol Vis Sci* **61**, 42
434. Millet, A. M. C., Coustham, C., Champigny, C., Botella, M., Demeilliers, C., Devin, A., Galinier, A., Belenguer, P., Bordeneuve-Guibe, J., and Davezac, N. (2023) OPA1 deficiency impairs oxidative metabolism in cycling cells, underlining a translational approach for degenerative diseases. *Dis Model Mech* **16**
435. Kao, S. H., Yen, M. Y., Wang, A. G., Yeh, Y. L., and Lin, A. L. (2015) Changes in Mitochondrial Morphology and Bioenergetics in Human Lymphoblastoid Cells With Four Novel OPA1 Mutations. *Invest Ophthalmol Vis Sci* **56**, 2269-2278
436. Lu, T. M., Tsai, J. Y., Chen, Y. C., Huang, C. Y., Hsu, H. L., Weng, C. F., Shih, C. C., and Hsu, C. P. (2014) Downregulation of Sirt1 as aging change in advanced heart failure. *J Biomed Sci* **21**, 57
437. Nadtochiy, S. M., Yao, H., McBurney, M. W., Gu, W., Guarente, L., Rahman, I., and Brookes, P. S. (2011) SIRT1-mediated acute cardioprotection. *Am J Physiol Heart Circ Physiol* **301**, H1506-1512
438. Planavila, A., Iglesias, R., Giralt, M., and Villarroya, F. (2011) Sirt1 acts in association with PPARalpha to protect the heart from hypertrophy, metabolic dysregulation, and inflammation. *Cardiovasc Res* **90**, 276-284
439. Nemoto, S., Fergusson, M. M., and Finkel, T. (2005) SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1alpha. *J Biol Chem* **280**, 16456-16460
440. Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., and Puigserver, P. (2005) Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113-118
441. Jackson, C. W., Xu, J., Escobar, I., Saul, I., Fagerli, E., Dave, K. R., and Perez-Pinzon, M. A. (2024) Resveratrol Preconditioning Downregulates PARP1 Protein to Alleviate

PARP1-Mediated Cell Death Following Cerebral Ischemia. *Transl Stroke Res* **15**, 165-178
442. Li, P., Liu, Y., Burns, N., Zhao, K. S., and Song, R. (2017) SIRT1 is required for mitochondrial biogenesis reprogramming in hypoxic human pulmonary arteriolar smooth muscle cells. *Int J Mol Med* **39**, 1127-1136
443. Ryu, D. R., Yu, M. R., Kong, K. H., Kim, H., Kwon, S. H., Jeon, J. S., Han, D. C., and Noh, H. (2019) Sirt1-hypoxia-inducible factor-1alpha interaction is a key mediator of tubulointerstitial damage in the aged kidney. *Aging Cell* **18**, e12904
444. Lu, C., Jiang, B., Xu, J., Zhang, X., and Jiang, N. (2023) Neferine protected cardiomyocytes against hypoxia/oxygenation injury through SIRT1/Nrf2/HO-1 signaling. *J Biochem Mol Toxicol* **37**, e23398
445. Ma, B., Guo, B., Chen, Z., and Li, Y. (2022) SIRT1 regulates hypoxia-induced oxidative stress in cardiomyocytes via PI3K/MTOR signaling. *Cell Mol Biol (Noisy-le-grand)* **68**, 48-53
446. Sanders, B. D., Jackson, B., and Marmorstein, R. (2010) Structural basis for sirtuin function: what we know and what we don't. *Biochim Biophys Acta* **1804**, 1604-1616
447. Dokmanovic, M., Clarke, C., and Marks, P. A. (2007) Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* **5**, 981-989
448. Gregoretti, I. V., Lee, Y. M., and Goodson, H. V. (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J Mol Biol* **338**, 17-31
449. Mao, Y., Zhang, J., Zhou, Q., He, X., Zheng, Z., Wei, Y., Zhou, K., Lin, Y., Yu, H., Zhang, H., Zhou, Y., Lin, P., Wu, B., Yuan, Y., Zhao, J., Xu, W., and Zhao, S. (2024) Hypoxia induces mitochondrial protein lactylation to limit oxidative phosphorylation. *Cell Res* **34**, 13-30
450. Yang, X., Chang, H. C., Tatekoshi, Y., Mahmoodzadeh, A., Balibegloo, M., Najafi, Z., Wu, R., Chen, C., Sato, T., Shapiro, J., and Ardehali, H. (2023) SIRT2 inhibition protects against cardiac hypertrophy and ischemic injury. *Elife* **12**
451. Wu, B., You, S., Qian, H., Wu, S., Lu, S., Zhang, Y., Sun, Y., and Zhang, N. (2021) The role of SIRT2 in vascular-related and heart-related diseases: A review. *J Cell Mol Med* **25**, 6470-6478
452. Tang, X., Chen, X. F., Wang, N. Y., Wang, X. M., Liang, S. T., Zheng, W., Lu, Y. B., Zhao, X., Hao, D. L., Zhang, Z. Q., Zou, M. H., Liu, D. P., and Chen, H. Z. (2017) SIRT2 Acts as a Cardioprotective Deacetylase in Pathological Cardiac Hypertrophy. *Circulation* **136**, 2051-2067
453. Luo, Y. X., Tang, X., An, X. Z., Xie, X. M., Chen, X. F., Zhao, X., Hao, D. L., Chen, H. Z., and Liu, D. P. (2017) SIRT4 accelerates Ang II-induced pathological cardiac hypertrophy by inhibiting manganese superoxide dismutase activity. *Eur Heart J* **38**, 1389-1398
454. Xiao, Y., Zhang, X., Fan, S., Cui, G., and Shen, Z. (2016) MicroRNA-497 Inhibits Cardiac Hypertrophy by Targeting Sirt4. *PLoS One* **11**, e0168078
455. Sadhukhan, S., Liu, X., Ryu, D., Nelson, O. D., Stupinski, J. A., Li, Z., Chen, W., Zhang, S., Weiss, R. S., Locasale, J. W., Auwerx, J., and Lin, H. (2016) Metabolomics-

assisted proteomics identifies succinylation and SIRT5 as important regulators of cardiac function. *Proc Natl Acad Sci U S A* **113**, 4320-4325
456. Boylston, J. A., Sun, J., Chen, Y., Gucek, M., Sack, M. N., and Murphy, E. (2015) Characterization of the cardiac succinylome and its role in ischemia-reperfusion injury. *J Mol Cell Cardiol* **88**, 73-81
457. Zhou, B., Xiao, M., Hu, H., Pei, X., Xue, Y., Miao, G., Wang, J., Li, W., Du, Y., Zhang, P., and Wei, T. (2021) Cardioprotective Role of SIRT5 in Response to Acute Ischemia Through a Novel Liver-Cardiac Crosstalk Mechanism. *Front Cell Dev Biol* **9**, 687559
458. Yu, L. M., Dong, X., Xue, X. D., Xu, S., Zhang, X., Xu, Y. L., Wang, Z. S., Wang, Y., Gao, H., Liang, Y. X., Yang, Y., and Wang, H. S. (2021) Melatonin attenuates diabetic cardiomyopathy and reduces myocardial vulnerability to ischemia-reperfusion injury by improving mitochondrial quality control: Role of SIRT6. *J Pineal Res* **70**, e12698
459. Li, X., Liu, L., Jiang, W., Liu, M., Wang, Y., Ma, H., Mu, N., and Wang, H. (2022) SIRT6 Protects Against Myocardial Ischemia-Reperfusion Injury by Attenuating Aging-Related CHMP2B Accumulation. *J Cardiovasc Transl Res* **15**, 740-753
460. Wang, X. X., Wang, X. L., Tong, M. M., Gan, L., Chen, H., Wu, S. S., Chen, J. X., Li, R. L., Wu, Y., Zhang, H. Y., Zhu, Y., Li, Y. X., He, J. H., Wang, M., and Jiang, W. (2016) SIRT6 protects cardiomyocytes against ischemia/reperfusion injury by augmenting FoxO3alpha-dependent antioxidant defense mechanisms. *Basic Res Cardiol* **111**, 13
461. Wang, Y., Zhang, S., Ma, Y., Xiang, A., Sun, H., Song, J., Yang, W., Li, X., and Xu, H. (2022) Melatonin protected against myocardial infarction injury in rats through a Sirt6-dependent antioxidant pathway. *Adv Clin Exp Med* **31**, 277-284
462. Shen, H., Holliday, M., Sheikh-Hamad, D., Li, Q., Tong, Q., Hamad, C. D., and Pan, J. S. (2021) Sirtuin-3 mediates sex differences in kidney ischemia-reperfusion injury. *Transl Res* **235**, 15-31
463. Barcena de Arellano, M. L., Pozdniakova, S., Kuhl, A. A., Baczko, I., Ladilov, Y., and Regitz-Zagrosek, V. (2019) Sex differences in the aging human heart: decreased sirtuins, pro-inflammatory shift and reduced anti-oxidative defense. *Aging (Albany NY)* **11**, 1918-1933
464. Mendirichaga, R., and Jacobs, A. K. (2020) Sex Differences in Ischemic Heart Disease-the Paradox Persists. *JAMA Cardiol* **5**, 754-756
465. Gery, I., and Handschumacher, R. E. (1974) Potentiation of the T lymphocyte response to mitogens. III. Properties of the mediator(s) from adherent cells. *Cell Immunol* **11**, 162-169
466. Miao, R., Jiang, C., Chang, W. Y., Zhang, H., An, J., Ho, F., Chen, P., Zhang, H., Junqueira, C., Amgalan, D., Liang, F. G., Zhang, J., Evavold, C. L., Hafner-Bratkovic, I., Zhang, Z., Fontana, P., Xia, S., Waldeck-Weiermair, M., Pan, Y., Michel, T., Bar-Peled, L., Wu, H., Kagan, J. C., Kitsis, R. N., Zhang, P., Liu, X., and Lieberman, J. (2023) Gasdermin D permeabilization of mitochondrial inner and outer membranes accelerates and enhances pyroptosis. *Immunity* **56**, 2523-2541 e2528

467. Ban, T., Ishihara, T., Kohno, H., Saita, S., Ichimura, A., Maenaka, K., Oka, T., Mihara, K., and Ishihara, N. (2017) Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin. *Nat Cell Biol* **19**, 856-863
468. Ban, T., Kohno, H., Ishihara, T., and Ishihara, N. (2018) Relationship between OPA1 and cardiolipin in mitochondrial inner-membrane fusion. *Biochim Biophys Acta Bioenerg* **1859**, 951-957
469. Raemy, E., Montessuit, S., Pierredon, S., van Kampen, A. H., Vaz, F. M., and Martinou, J. C. (2016) Cardiolipin or MTCH2 can serve as tBID receptors during apoptosis. *Cell Death Differ* **23**, 1165-1174
470. Liu, L. X., Zheng, X. H., Hai, J. H., Zhang, C. M., Ti, Y., Chen, T. S., and Bu, P. L. (2024) SIRT3 regulates cardiolipin biosynthesis in pressure overload-induced cardiac remodeling by PPARgamma-mediated mechanism. *PLoS One* **19**, e0301990
471. Cheung, K. G., Cole, L. K., Xiang, B., Chen, K., Ma, X., Myal, Y., Hatch, G. M., Tong, Q., and Dolinsky, V. W. (2015) Sirtuin-3 (SIRT3) Protein Attenuates Doxorubicin-induced Oxidative Stress and Improves Mitochondrial Respiration in H9c2 Cardiomyocytes. *J Biol Chem* **290**, 10981-10993
472. Zhang, Y., Bharathi, S. S., Rardin, M. J., Uppala, R., Verdin, E., Gibson, B. W., and Goetzman, E. S. (2015) SIRT3 and SIRT5 regulate the enzyme activity and cardiolipin binding of very long-chain acyl-CoA dehydrogenase. *PLoS One* **10**, e0122297
473. Chabi, B., Fouret, G., Lecomte, J., Cortade, F., Pessemesse, L., Baati, N., Coudray, C., Lin, L., Tong, Q., Wrutniak-Cabello, C., Casas, F., and Feillet-Coudray, C. (2018) Skeletal muscle overexpression of short isoform Sirt3 altered mitochondrial cardiolipin content and fatty acid composition. *J Bioenerg Biomembr* **50**, 131-142
474. Mao, Q., Liang, X. L., Zhang, C. L., Pang, Y. H., and Lu, Y. X. (2019) LncRNA KLF3-AS1 in human mesenchymal stem cell-derived exosomes ameliorates pyroptosis of cardiomyocytes and myocardial infarction through miR-138-5p/Sirt1 axis. *Stem Cell Res Ther* **10**, 393
475. Ma, Z., Tang, P., Dong, W., Lu, Y., Tan, B., Zhou, N., Hao, J., Shen, J., and Hu, Z. (2022) SIRT1 alleviates IL-1beta induced nucleus pulposus cells pyroptosis via mitophagy in intervertebral disc degeneration. *Int Immunopharmacol* **107**, 108671
476. Gong, Y., Tang, N., Liu, P., Sun, Y., Lu, S., Liu, W., Tan, L., Song, C., Qiu, X., Liao, Y., Yu, S., Liu, X., Lin, S. H., and Ding, C. (2022) Newcastle disease virus degrades SIRT3 via PINK1-PRKN-dependent mitophagy to reprogram energy metabolism in infected cells. *Autophagy* **18**, 1503-1521
477. Li, R., Wang, Z., Wang, Y., Sun, R., Zou, B., Tian, X., Liu, D., Zhao, X., Zhou, J., Zhao, Y., and Yao, J. (2023) SIRT3 regulates mitophagy in liver fibrosis through deacetylation of PINK1/NIPSNAP1. *J Cell Physiol* **238**, 2090-2102
478. Yu, W., Gao, B., Li, N., Wang, J., Qiu, C., Zhang, G., Liu, M., Zhang, R., Li, C., Ji, G., and Zhang, Y. (2017) Sirt3 deficiency exacerbates diabetic cardiac dysfunction: Role of Foxo3A-Parkin-mediated mitophagy. *Biochim Biophys Acta Mol Basis Dis* **1863**, 1973-1983
479. Miyazaki, N., Shiratori, R., Oshima, T., Zhang, Z., Valencia, R., Kranrod, J., Fang, L., Seubert, J. M., Ito, K., and Aoki, S. (2022) PINK1-dependent and Parkin-independent

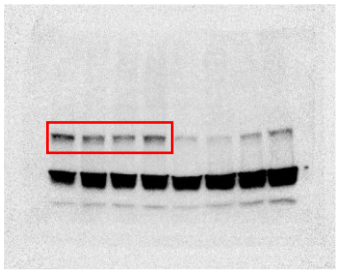
mitophagy is involved in reprogramming of glycometabolism in pancreatic cancer cells. *Biochem Biophys Res Commun* **625**, 167-173
480. Kumar, A., and Shaha, C. (2018) SESN2 facilitates mitophagy by helping Parkin translocation through ULK1 mediated Beclin1 phosphorylation. *Sci Rep* **8**, 615
481. Geisler, S., Holmstrom, K. M., Skujat, D., Fiesel, F. C., Rothfuss, O. C., Kahle, P. J., and Springer, W. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* **12**, 119-131
482. Harper, J. W., Ordureau, A., and Heo, J. M. (2018) Building and decoding ubiquitin chains for mitophagy. *Nat Rev Mol Cell Biol* **19**, 93-108
483. Bouhidel, J. O., Wang, P., Siu, K. L., Li, H., Youn, J. Y., and Cai, H. (2015) Netrin-1 improves post-injury cardiac function in vivo via DCC/NO-dependent preservation of mitochondrial integrity, while attenuating autophagy. *Biochim Biophys Acta* **1852**, 277-289
484. Kheyar, A., Ahnou, N., Ahmed-Belkacem, A., Hulin, A., Pressiat, C., Ghaleh, B., Guichou, J. F., Morin, D., Pawlotsky, J. M., and Teixeira-Clerc, F. (2023) The novel cyclophilin inhibitor C105SR reduces hepatic ischaemia-reperfusion injury via mitoprotection. *JHEP Rep* **5**, 100876
485. Endo, T., Samokhvalov, V., Darwesh, A. M., Khey, K. M. W., El-Sherbeni, A. A., Ei-Kadi, A. O. S., Machida, T., Hirafuji, M., and Seubert, J. M. (2018) DHA and 19,20-EDP induce lysosomal-proteolytic-dependent cytotoxicity through de novo ceramide production in H9c2 cells with a glycolytic profile. *Cell Death Discov* **4**, 29
486. Zulkifli, N. D., and Zulkifle, N. (2023) Insight from sirtuins interactome: topological prominence and multifaceted roles of SIRT1 in modulating immunity, aging and cancer. *Genomics Inform* **21**, e23
487. Falck, J. R., Kodela, R., Manne, R., Atcha, K. R., Puli, N., Dubasi, N., Manthati, V. L., Capdevila, J. H., Yi, X. Y., Goldman, D. H., Morisseau, C., Hammock, B. D., and Campbell, W. B. (2009) 14,15-Epoxyeicosa-5,8,11-trienoic acid (14,15-EET) surrogates containing epoxide bioisosteres: influence upon vascular relaxation and soluble epoxide hydrolase inhibition. *J Med Chem* **52**, 5069-5075

APPENDIX

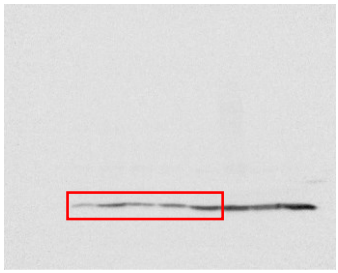
Uncropped representative immunoblot images for figures (red rectangle represents target bands)

(Figure 3.3)

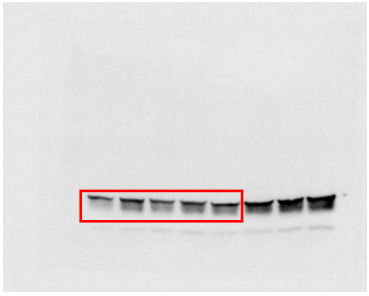
SDH-A



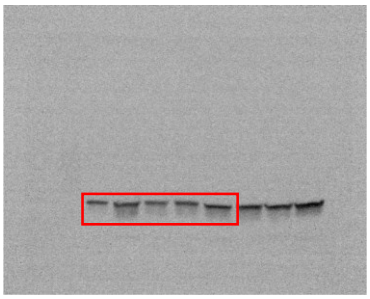
COX-IV



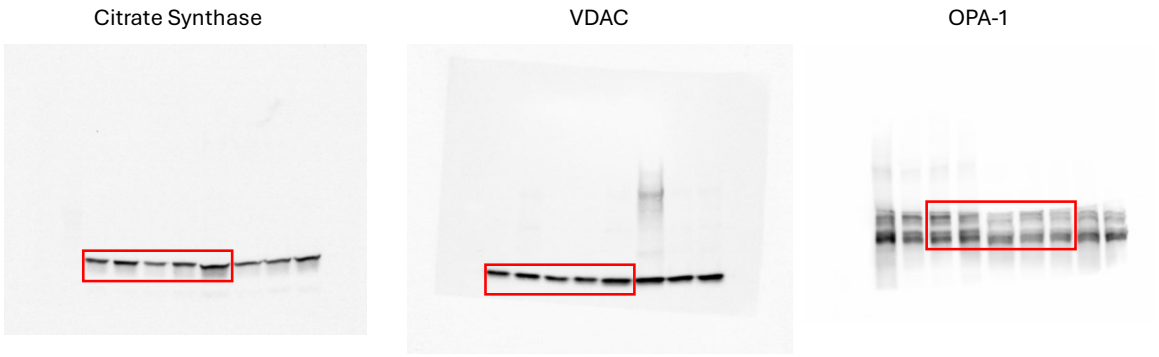
Citrate synthase



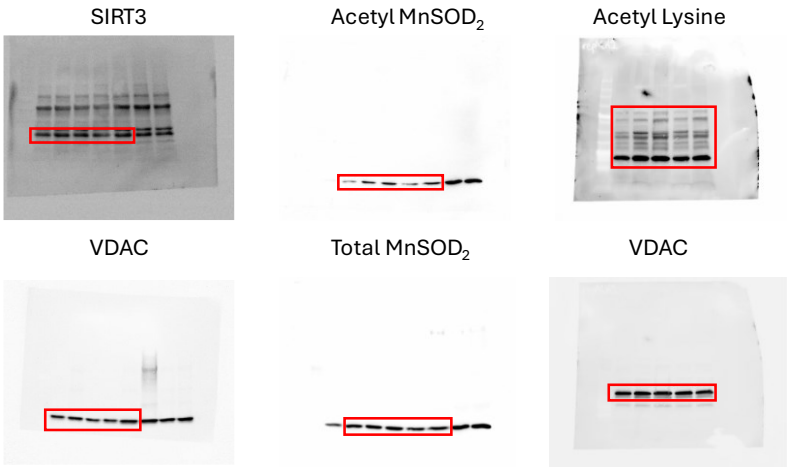
Citrate synthase



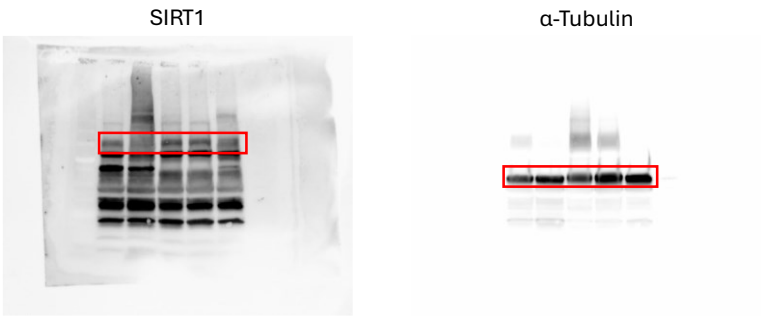
(Figure 3.4)



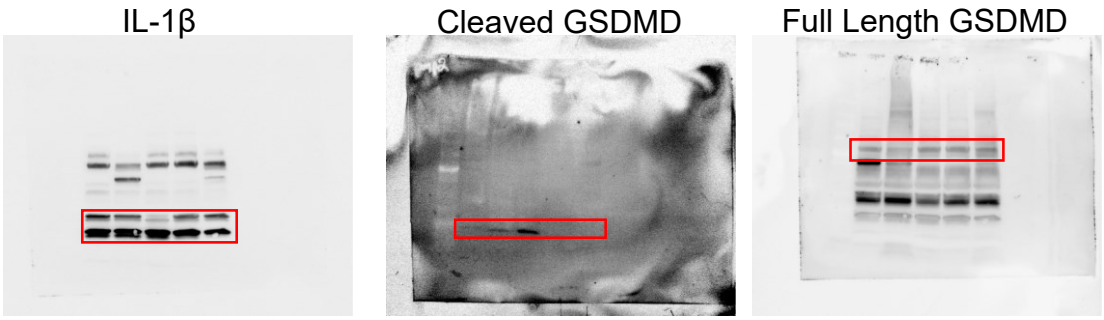
(Figure 3.5)



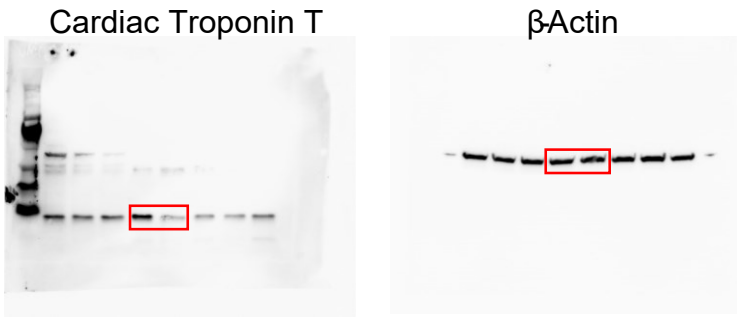
(Figure 3.6)



(Figure 3.7)



(Figure 3.8)



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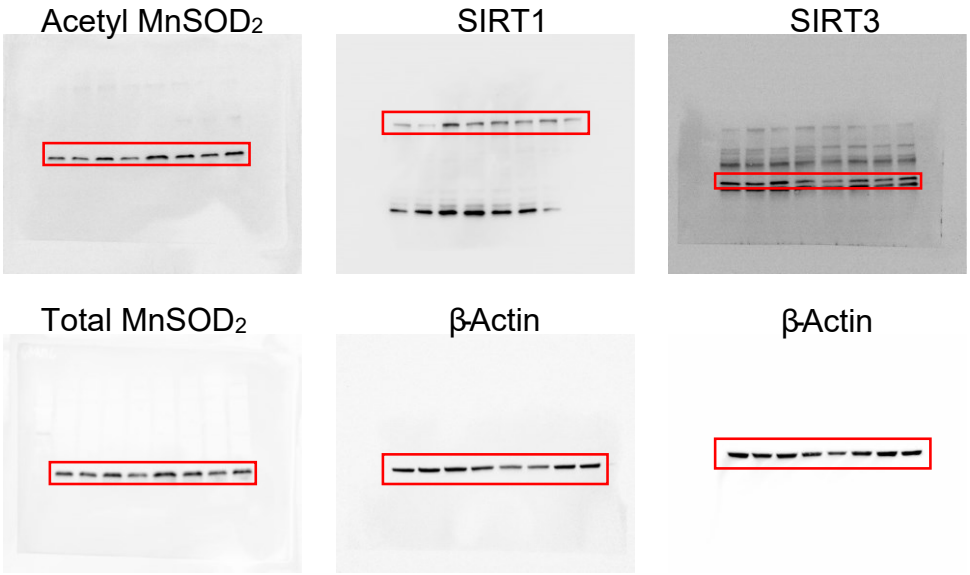
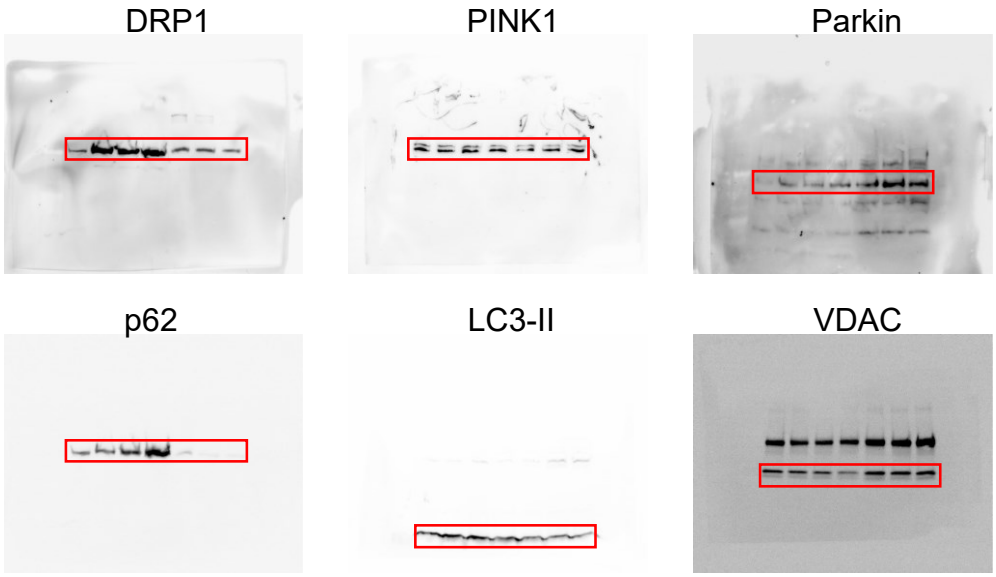


(Figure 3.11)



(Figure 3.15)