Cardioprotective effects of n-3 and n-6 PUFA derived epoxylipids against age-related cardiac alterations

by

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ABSTRACT

Age-associated changes leading to a decline in cardiac structure and function contribute to the increased susceptibility and incidence of cardiovascular diseases (CVD) in elderly individuals. A steady decline in mitochondrial function is recognized as an important biological consequence found in the aging heart which contributes to increased cellular stress and age-related CVD pathogenesis. There is growing evidence indicating CYP450 epoxygenase-mediated metabolites of n-3 and n-6 polyunsaturated fatty acids are active lipid mediators regulating cardiac homeostasis. These epoxy metabolites are rapidly hydrolyzed and inactivated by the soluble epoxide hydrolase (sEH). Both genetic deletion and pharmacological inhibition of sEH has been demonstrated to mediate cardioprotective, anti-inflammatory and anti-hypertensive responses, as well limit mitochondrial injury. In this thesis, we characterized cardiac differences in young and aged sEH null mice compared to the corresponding wild-type (WT) mice. Significant increased cardiac hypertrophy was observed in all aged groups except female sEH null mice. Sirtuin-3 (Sirt-3) activity significantly decreased over aging in WT both males and females associated with increased expression level of acetyl manganese superoxide dismutase (acetyl Mn-SOD). Genetic deletion of sEH preserved Sirt-3 activity coupled with lower level of acetyl Mn-SOD in the hearts of both female and male mice. Consistent with these changes, the activity level of SOD significantly decreased in WT animals but was preserved in aged sEH null animals. There was an age-related disruption in mitochondrial ultrastructure in WT animals which was attenuated is sEH null mice. Together these data demonstrate the sexual dimorphic pattern in beneficial effects of genetic deletion of sEH in limiting age-related cardiac alterations.

PREFACE

The thesis is an original work by Hedieh Keshavarz-Bahaghighat. All animal experiments were carried out in accordance with protocols that were evaluated and approved by the University of Alberta Animal Care and Use Committee. Chapter 1 includes content previously published in (Hedieh Keshavarz-Bahaghighat, Ahmed M Darwesh, Deanna K Sosnowski, John M Seubert; Mitochondrial Dysfunction and Inflammaging in Heart Failure: Novel Roles of CYP-Derived Epoxylipids. Cells, 2020 Jun 27;9(7)). Chapter 2 includes content previously published in (K Lockhart Jamieson, Hedieh Keshavarz-Bahaghighat, Ahmed M Darwesh, Deanna K Sosnowski, John M Seubert; Age and Sex Differences in Hearts of Soluble Epoxide Hydrolase Null Mice. Front physiol, 2020 Feb 7;11:48.). In this manuscript, KLJ and HKB had equal contribution and are both first co-authors.

DEDICATION

Dedicated to the victims of flight PS752

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I share this accomplishment with all of you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AEPU	Adamantan-3-(5-(2-(2-ethylethoxy) ethoxy) pentyl) urea
ALA	α-linolenic acid
Ang II	Angiotensin II
c-AUCB	cis-4-[4-(3-adamantan-1-yl-ureido) cyclohexyloxy] benzoic acid
CVD	Cardiovascular disease
CS	Citrate synthase
COX	Cyclooxygenases
CYP	Cytochrome P450
COX-I	Cytochrome c oxidase subunit I
DCM	Dilated cardiomyopathy
DHA	Docosahexaenoic acid
DiHOME	Dihydroxyoctadecenoic acid
DiHDPA	Dihydroxydocasapentaneoic acid
DHEQ	Dihydroxyeicosatetraenoic acid
Drp-1	Dynamin related protein 1
EDP	Epoxydocosapentaenoic acids
EET	Epxoyeicosatrienoic acids
EEQ	Epoxyeicosatetraenoic acid
EPA	Eicosapentaenoic acid
EpOME	Epoxyoctadecamonoenic acid
Fis1	Fission protein 1
HF	Heart failure

HW	Heart weight
HO-1	Hem-oxygenase 1
IL-1β	Interlukin-1β
LA	Linoleic acid
LAD	Left anterior descending artery
LPS	Lipopolysaccharide
LOX	Lipoxygenases
LV	Left ventricular
MCAD	Medium chain acyl-CoA dehydrogenase
MCAT	Mitochondrial catalase
MDA	Malondialdehyde
mEH	Microsomal epoxide hydrolase
Mfn	Mitofusin
MI	Myocardial infarction
MnSOD	Manganese superoxide dismutase
MPTP	Mitochondrial permeability transition pore
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
NUDSA	(S)-2-(11-(nonyloxy) undec-8(Z)-enamido) succinic acid
OPA1	Optic atrophy 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PUFA	Polyunsaturated fatty acids
RCP	Respiratory control ratio
ROS	Reactive oxygen species
SA-β-gal	Senescence-associated β-galactosidase

sEH	Soluble epoxide hydrolase
Sir2	Silent information regulator 2
Sirt	Sirtuin
SOD	Superoxide dismutase
TAC	Transverse aortic constriction
TGF-β	Tumor growth factor β
TL	Tibia length
Trx	Thioredoxin
Txnip	Thioredoxin interacting protein
UA-8	13-(3-propylureido) tridec-8-enoic acid
WT	Wild-type

Chapter 1: Introduction

This chapter includes content previously published in the following review paper:

Hedieh Keshavarz-Bahaghighat, Ahmed M Darwesh, Deanna K Sosnowski, John M Seubert; Mitochondrial Dysfunction and Inflammaging in Heart Failure: Novel Roles of CYP-Derived Epoxylipids. Cells, 2020 Jun 27;9(7)

1.1 General background

Aging is a key determinant of cardiovascular health, as evidenced from an exponential increase in the prevalence of cardiovascular disease (CVD) in the geriatric population [1]. Aging hearts can be characterized by overall decreased function, reduced cardiac reserve capacity, structural remodeling and electrical dysfunction [2, 3]. The extent and duration of exposure to extrinsic risk factors linked to development of CVD, such as hypertension, diabetes and smoking can contributing to increased frequency and severity of cardiovascular morbidity and mortality in aged individuals [4, 5]. In addition, naturally occurring biological aging events lead to a slowly progressive deterioration in cardiac structure and function even in the absence of pathologic risk factors [6]. In essence, the age-associated extrinsic and intrinsic changes converge to accelerate a deterioration of cardiac function and structure contributing to the increased susceptibility and incidence of CVD in elderly.

Studies in experimental animal models and human hearts suggest that mitochondria play a central role in the aging process and abnormalities in mitochondrial function and structure are considered major drivers of age-associated cardiac dysfunction [3, 7, 8]. In healthy myocardium, mitochondria provide up to 90% of energy demand of the beating heart by mediating electron transportation to generate ATP on a beat-to-beat basis [9]. Mismatch between ATP supply and demand attributed to mitochondrial dysfunction has been historically considered as the primary mechanism linking mitochondria to cardiovascular diseases [10-12]. However, the role of mitochondria is now increasingly recognized to reach far beyond a failed powerhouse [13, 14]. Defective

mitochondrial reactive oxygen species (ROS) handling has emerged as a central factor in pathogenesis of a wide variety of cardiovascular dysfunctions, including cardiac aging and heart failure (HF) [15]. Enhancing our understanding of signaling pathways interwoven with mitochondrial events in the process of cardiac aging remains important, notably toward developing more promising therapeutics for age-associated pathologies, including HF.

1.2 Aging mitochondria contribute to age-related cardiac alterations

Given the immense energetic cost of cardiac electrical and mechanical function and the limited capacity for energy storage, the heart mainly relies on mitochondria as a steady energy supply [11, 16]. Approximately 95% of cardiac ATP is produced by mitochondria through oxidative phosphorylation. However, the importance of cardiac mitochondrial health and function is now increasingly recognized to reach beyond ATP synthesis [17]. Mitochondria play a central role in a myriad of cellular processes, such as oxidative stress homeostasis, biosynthetic pathways, signaling and programmed cell death [18]. It has long been appreciated that aging is accompanied by a decline in mitochondrial function and quality contributing to a wide variety of age-related diseases, including HF [19, 20]. Aged cardiomyocytes show extensive mitochondrial abnormalities, including enlarged organelles, loss of cristae, reduction in ATP synthesis, impaired dynamics and increased ROS production [8, 17, 21, 22]. Historically, numerous studies have proposed the detrimental alterations in mitochondrial function and structure play a central role in

myocardial hypertrophy, fibrosis and consequently transition to HF [23, 24]. The role mitochondria have in activating various age-related signalling pathways has led to new concepts for their involvement in aging and age-related diseases.

1.3 Mitochondria and oxidative stress theory of aging

Dysregulated ROS production and impaired antioxidant defense have been implicated in a host of cardiovascular dysfunctions, including cardiac aging and HF [25]. Age-associated oxidative stress has been demonstrated in clinical studies, evidenced by depleted glutathione, impaired superoxide dismutase (SOD) and an increase in malondialdehyde levels in elderly individuals [26-28]. Excessive production of ROS triggers numerous adverse effects leading to cell dysfunction, lipid peroxidation and DNA mutagenesis ultimately resulting in irreversible cell damage and death [29]. Conversely, overexpression of antioxidant molecules, including mitochondrial thioredoxin (Trx) and catalase, has been suggested to extend the life span in animal models [30, 31]. Over expression of human Trx in mice protected murine bone morrow cells from UVC-induced oxidative stress and improved telomerase activity associated with increased maximum life span [30]. Elevated mitochondrial catalase (MCAT) activity in mouse hearts attenuated the severity of age-induced cardiomyopathy and arthrosclerosis and reduced oxidative damage to cardiac total DNA resulting in a longer median life span in MCAT over-expressed mice compared to their wild-type (WT) counterparts [31].The occurrence of increased oxidative stress in aging is thought to be a driver of inflammation activation [32]. Several underlying novel pathways regulating age-associated oxidative stress have

been elucidated, among which mitochondrial ROS generation is of particular importance in the setting of age-associated inflammation activation [33-35].

Mitochondrial respiratory chain serves as a major source of ROS production where leakage of single electrons are transferred to molecular oxygen forming superoxide anions [36]. Due to the proximity to the electron transport chain, mitochondrial DNA (mtDNA) is highly susceptible to ROS-mediated damage, leading to further mitochondrial dysfunction [37]. The vicious cycle between mitochondrial damage and further overproduction of ROS causes dysregulation of various cellular pathways, including inflammation, apoptosis and eventually, resulting in cardiac functional decline [38, 39].

Based on an oxidative stress hypothesis in aging, both increased levels of ROS and a decline in efficiency of antioxidant systems contribute to age-associated progressive degeneration in cardiac function and structure [40]. Mitochondrial thioredoxin system (Trx 2), localized to the mitochondrial matrix, is a major free radical scavenger providing a primary defense against mitochondrial ROS. Trx is found at the highest levels in metabolically active tissues, including cardiac cells playing a major role in the apoptotic pathway by protecting cells against oxidative stress damage [41]. Thioredoxin interacting protein (Txnip) has been identified as a tumor suppressor protein with a primary role of inhibiting antioxidant activity of Trx via direct interaction [42]. Upon stress conditions, Txnip is shuttled into mitochondria and inhibits the antioxidant activity of Trx 2 leading to increase mitochondrial ROS production and leakage [43]. Both lowered levels of Trx 2 and increased levels of Txnip have been documented in the process of aging and age-related diseases contributing to unbalanced oxidative stress, a hallmark of aging [41, 44, 45]. Trx 2 mRNA level was detected to decrease in auditory cortex in a mimetic aging rat

model induced by D-galactose associated with significantly increased Txnip mRNA resulting in decreased SOD activity and increased malondialdehyde (MDA) level [41]. Increased Txnip expression has been also documented in isolated primary T cells from elderly patients (>55 years old) compared to young (20-25 years old) individuals accompanied by lower level of Trx activity suggesting that increased Txnip may contribute to age-related pre-oxidative status during aging [44]. While Txnip was initially characterized as a key regulator in cellular redox signaling pathways, evidence suggests the function of Txnip goes beyond classical redox biology. Recent data indicate Txnip might play a key role in aging by linking oxidative stress and inflammation activation [20, 46]. Age-dependent upregulation of Txnip leads to accumulation of ROS, increased oxidative stress and perturbation of cellular redox equilibrium[44]. Studies on murine models of diabetic nephropathy and diabetic patients have revealed that increased production of ROS , particularly mitochondrial ROS, results in Txnip association and activation of caspase-1 and release of interlukin-1β (IL-1β) [47, 48]. Together, it seems that mitochondrial shuttling of Txnip may affect mitochondrial dysfunction and oxidation of mtDNA, leading to increased oxidative stress level in the aging heart.

1.4 Impaired mitochondrial dynamics in cardiac aging

Mitochondria are dynamic organelles constantly undergoing fission and fusion events in response to energy demand and cellular stress. The balanced fission and fusion events under basal conditions is responsible for maintaining mitochondrial morphology and metabolism [17]. Key proteins regulating fission include dynamin related protein 1

(Drp-1) and fission protein 1 (Fis1), while mitofusin 1 and 2 (Mfn-1 and 2) and optic atrophy 1 (OPA1) are involved in mitochondrial fusion in mammals. Altered expression or activation of mitochondrial dynamic proteins have been implicated in the pathogenesis of cardiac diseases [9]. Cardiac specific ablation of Drp-1 gene in mice inhibits mitochondrial fission, resulting in mitochondrial enlargement, increased mitochondrial permeability transition pore (MPTP) opening, apoptosis, and ultimately, lethal dilated cardiomyopathy (DCM) [49]. Interrupting mitochondrial fusion with deletion of Mfn-1 and Mfn-2 genes in mice, also leads to progressive and lethal DCM, primarily due to disrupted mitochondrial structure and respiratory chain function [50]. Evidence is suggesting the imbalance between mitochondrial fission and fusion during aging has a role in age-related CVD via compromising mitochondrial integrity [8, 51, 52]. Reduced fission and/or increased fusion have been shown to be associated with elongated, hyper-fused mitochondria in aged tissues [53-55]. The ratio of Mfn-2 and Drp-1, as an indicator of the balance in fission/fusion, was significantly increased in skeletal muscle of aged mice associated with more elongated mitochondria which may partially explain the increased susceptibility of the aged mitochondria to stress [53]. Similarly, mitochondria from aged C. elegans showed significantly enlarged and swollen ultrastructure accompanied by decreased oxygen consumption, increased carbonylated protein and decreased mitochondrial SOD activity [55]. While an elongated morphology is associated with accumulation of dysfunctional mitochondria in aged tissues, promoting Drp-1 mediated fission in midlife in Drosophila improves mitochondrial respiratory function and structure, prolongs life span and delays age-related pathologies [56]. Concomitant interruption of

fission and fusion processes can accelerate mitochondrial senescence and result in the accumulation of dysfunctional mitochondria, contributing to the development of HF [38].

1.5 Sirtuin-3, a key regulator in mitochondria and aging

The sirtuin family consists of seven mammalian members (Sirt 1-7) homologous to yeast silent information regulator 2 (Sir2). Sirtuins are classified as NAD^+- Dependent histone deacetylases which act as an acceptor molecule for the acetyl group removed from their target proteins [57]. Sirtuins differ in their subcellular localization, expression patterns, and biological functions [58]. Sirt-1, Sirt-6 and Sirt-7 are nuclear proteins, Sirt-2 is predominantly cytoplasmic and Sirt-3, Sirt-4 and Sirt-5 are localized to mitochondria [57]. While Sirt 1-3 are well recognized for their strong deacetylase activity, Sirt 4-7 are reported to have no or weak deacetylase activity and exert other types of enzymatic activities, including, demyristoylase, depalmitoylase, and ADP-ribosyltransferase [59]. Sirtuin proteins are increasingly known as important regulators of a wide variety of physiological and pathological processes such as metabolism, stress response, apoptosis, inflammation, and aging [60].

Among the three mitochondrial sirtuins, Sirt-3 is the primary deacetylase playing a central role in regulating a diverse array of mitochondrial functions suggesting that Sirt-3 is a mitochondrial fidelity protein [61, 62]. Genetic deletion of Sirt-3 resulted in hyper-lysin acetylation of mitochondria, unbalanced oxidative stress status, decreased level of basal ATP, and reduction in the activity of mitochondrial complex I of the electron transport chain [63]. Sirt-3 is expressed highly in organs with high metabolic activity,

including the heart and is increasingly recognized as a key regulator in preserving mitochondrial integrity and improving cardiac function [64]. Both overexpression of Sirt-3 and treatment with Sirt-3 activator, resveratrol, attenuated triptolide-induced cardiotoxicity and mitochondrial dysfunction via decreasing oxidative stress and closing MPTP [65]. Isolated perfused hearts from Sirt-3 null mice showed worse cardiac dysfunction during post-ischemic recovery compared to their WT counterparts coupled with increased MPTP opening and ROS generation [66]. Moreover, pharmacological activation of Sirt-3 significantly attenuated ischemia-perfusion induced necrosis and apoptosis associated with decreased ROS level resulted from Sirt-3mediated deacetylation [67]. Restoring Sirt-3 activity through balancing the $NADH/NAD^+$ ratio attenuated cardiac hypertrophy in murine hearts subjected to transverse aortic constriction (TAC) [68]. While Sirt-3 deficient mice appeared normal at birth, they developed cardiac hypertrophy and interstitial fibrosis at 8 weeks of age and showed significantly increased cardiac stress and sensitivity to TAC induced cardiac injury [69].

Both mitochondrial dysfunction and increased ROS are major hallmarks of cardiac aging predisposing the elderly population toward developing age-related CVDs [70]. Sirt-3 has emerged as a possible key regulator of aging, due to its mitochondrial localization and decreased expression with age contributing to mitochondrial dysfunction [71, 72]. Aged murine hematopoietic stem cells showed downregulated levels of Sirt-3 mRNA with increased oxidative stress and accumulation of damaged mitochondria associated with unbalanced stress-responsive mitochondrial homeostasis [73]. Sirt-3 expression level in veins of old rat was significantly lower than their young counterparts coupled with neointima hyperplasia and deteriorated hemodynamics in vein grafts. Interestingly,

increased level of sirt-3 via Ad transfection significantly inhibited vascular smooth muscle proliferation and neointima hyperplasia, and improved hemodynamics [74]. Aged transgenic mice with whole-body Sirt-3 overexpression, showed reduced fibrotic markers, including Tumor growth factor β (TGF-β) and reduced cardiac fibrosis compared to their aged-matched WT counterparts [75]. Among various Sirt-3 target proteins, manganese superoxide dismutase (MnSOD) is of great importance in the context of aging as altered function of MnSOD can have remarkable consequences on mitochondrial function due to oxidative damage resulting in the development of various age-related diseases [70, 76]. MnSOD is a primary mitochondrial anti-oxidant enzyme and its principal physiological function is to provide a defense mechanism against deleterious effects of ROS [77]. Sirt-3 plays a significant role in regulating MnSOD enzymatic activity by directly deacetylating lysin residues leading to MnSOD activation [78]. Sirt-3 deficient and aged WT murine hearts showed similar phenotype towards ischemia-reperfusion injury resulting in an increased infarct size and worsened rate pressure product. Furthermore, mitochondrial protein acetylation was significantly increased in both Sirt-3 deficient and aged WT mice associated with decreased activity of MnSOD [79]. Sirt-3 depleted mouse aortas showed significantly higher level of acetylated MnSOD, as well as increased cell-senescence markers compared with WT mice, including p21 and senescence-associated β-galactosidase (SA-β-gal) leading to exaggerated end-organ inflammation [80]. In D-galactose mimetic aging rats, Sirt-3 expression and MnSOD activity were both significantly decreased in central auditory cortex leading to elevated ROS levels. Consequently, increased oxidative stress and subsequent mitochondrial dysfunction and abnormal ultrastructure led to inappropriate cellular apoptosis and age-related dysfunction

of the central auditory system [81]. Together, current data have provided strong evidence that Sirt-3 is a main regulator of mitochondrial metabolism and function, including maintaining the balance between mitochondrial anti-oxidant defense and ROS production. Considering the central role of mitochondria-derived ROS in aging and age-related cardiac diseases, therapeutic approaches aim to improve Sirt-3 activity seem to be promising.

1.6 N-3 and N-6 polyunsaturated fatty acids (PUFAs)

Long chain polyunsaturated fatty acids (PUFA) are essential fatty acids obtained from dietary sources which are required for cellular organelles, such as phospholipid membranes and serve as precursors to numerous bioactive lipid mediators [82]. Linoleic acid (LA) is the primary source of N-6 PUFA, which is converted to arachidonic acid (AA), while α-linolenic acid (ALA) is considered the main N-3 PUFA precursor [83]. Inside the body, the conversion of ALA and LA to their corresponding downstream metabolites happens through multiple elongation and desaturation steps [84]. ALA can be converted into eicosapentaenoic acid (EPA) which can be further metabolized to yield docosahexaenoic acid (DHA); however, the conversion is very limited in humans [85]. Since the N-3 and N-6 PUFAs compete for the same metabolic pathways, the dietary N-3:N-6 PUFA ratio plays a critical role in normal tissue function and development [83]. A typical western diet provides higher levels of LA resulting in predominant generation of LA derived eicosanoids, which results in an unbalanced ratio correlating with the etiology of many diseases, including CVD [84, 86].

Emerging evidence demonstrates the epoxy, hydroxyl and diol metabolites derived from N-3 and N-6 PUFA metabolism have important properties. The metabolism of N-3 and N-6 PUFA occurs primarily through three enzymatic systems, cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP) enzymes, into a plethora of bioactive metabolites. Various members of the CYP superfamily are capable of metabolizing N-3 and N-6 PUFAs into bioactive lipid mediators (Figure 1) [87, 88].

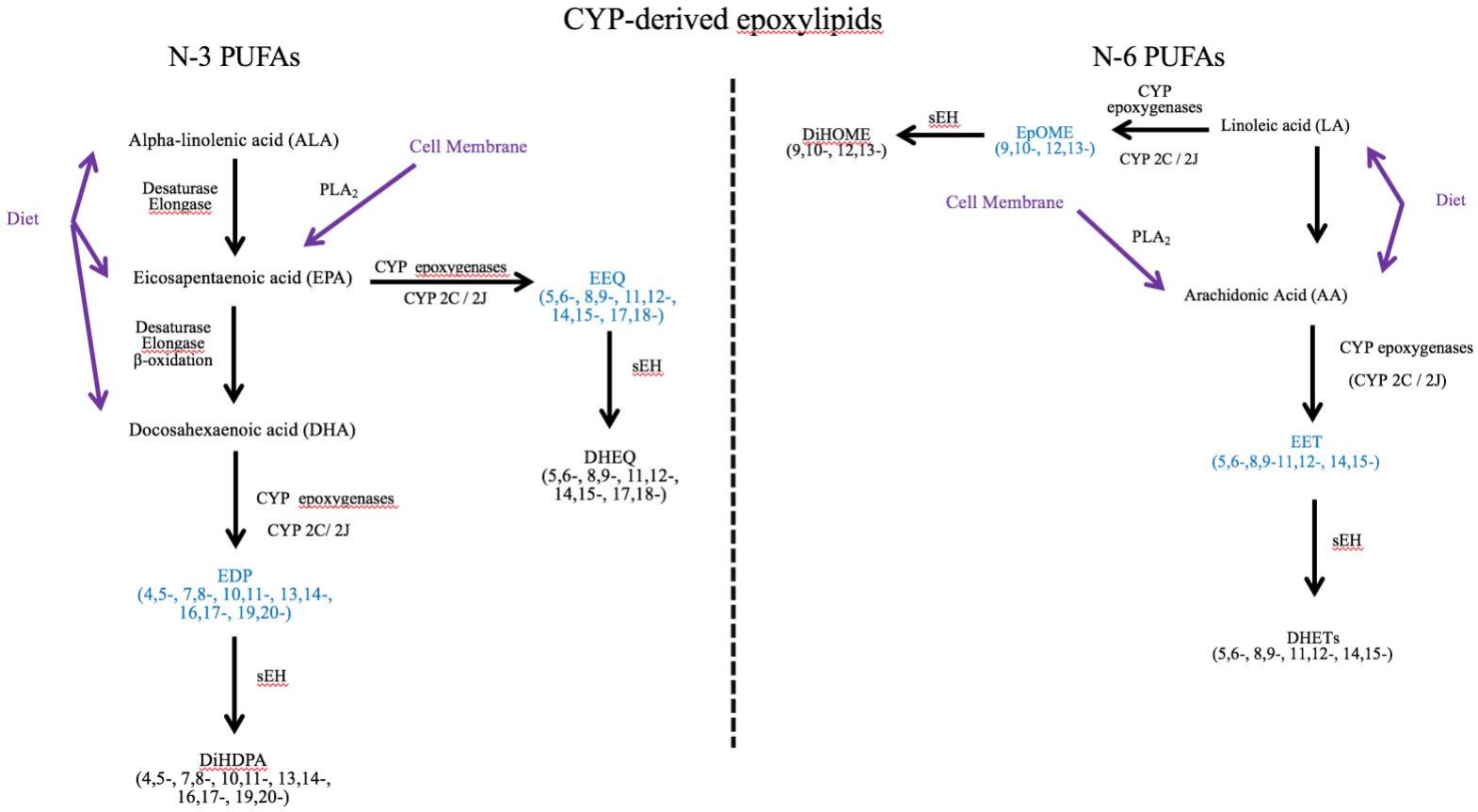

Figure 1.1. Overview of metabolic pathway of CYP-derived epoxylipids: LA, AA, ALA, EPA, and DHA are essential dietary fatty acids and are found in membrane phospholipids. AA: arachidonic acid; ALA: α-linolenic acid; CYP: cytochrome p450. DiHOME: dihydroxyoctadecenoic acid; DHA: docosahexaenoic acid; DiHDPA: dihydroxydocosapentaneoic acid; DHEQ: dihydroxyeicosatetraenoic acid; DHET: dihydroxyeicosatrienoic acid; EET: epoxyeicosatrienoic acid; EDP: epoxydocosapentaenoic acid; EEQ: epoxyeicosatetraenoic acid; EpOME: epoxyoctadecamonoenic acid; LA: linoleic acid; PUFA: poly unsaturated fatty acid, sEH: soluble epoxide hydrolase.

In the cardiovascular system, CYP2J and CYP2C isozymes are major epoxygenases responsible for converting AA, a N-6 PUFA, into four regioisomeric epxoyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) by olefin epoxidation [89, 90]. As well, converting the N-3 PUFAs, EPA into 5 regioisomeric epoxyeicosatetraenoic acids (5,6-, 8,9-, 11,12-, 14,15-, 17,18-EEQ) and DHA into 6 regioisomeric epoxydocosapentaenoic acids (4,5-, 7,8-, 10,11-, 13,14-, 16,17-, 19,20-EDP) [91]. In the heart, EETs act as the key lipid mediators, regulating cellular mechanisms, including mitochondrial quality control, apoptosis, and inflammatory pathways [92-94]. Although little is known about the exact mechanisms of cardioprotective effects of N-3 PUFAs, recent evidence demonstrates that CYP-derived epoxy metabolites possess cardioprotective effects [95, 96]. Most N-3 and N-6 epoxylipids, including EETs, EDP and EEQ, have a short half-life and are rapidly metabolized to their corresponding less active diol metabolites by the enzyme soluble epoxide hydrolase (sEH) [88, 97]. sEH belongs to epoxide hydrolase family of enzymes and is primarily responsible for detoxifying highly reactive epoxides via adding a molecule of water leading to production of more stable and less reactive intermediate metabolites [98]. Epoxides are well-established carcinogenetic molecules causing cancer and organ damage via interacting with various cellular macromolecules, particularly DNA and proteins. Hence, sEH , as one of the key epoxide detoxifying enzymes, regulates several physiological and pathophysiological pathways via its metabolic activities [99]. In mammalians, kidney and liver have highest level of sEH activity and expression level followed by other organs and tissues, including the lung and heart [100]. Although the molecular mechanisms of sEH regulation is poorly understood, it has been shown that peroxisome proliferator activated

receptor α (PPAR- α) agonists can induce the expression of sEH [101]. A decrease in sEH expression has been reported in various malignancies, including liver, kidney, colon, and prostate cell carcinoma [102]. The decrease in sEH expression leading to loss of essential hydrolase activity further contributes to DNA damage, and promoting tumor growth and carcinogenesis [103]. Besides the diminished hydrolase activity, overaccumulation of EETs following decreased sEH expression is also responsible for angiogenesis and tumor growth seen with sEH inhibitors [104].

Over the past decade, experimental studies have demonstrated EETs mediate a myriad of cellular and metabolic pathways, which are cardioprotective toward several pathologies including, myocardial infarction (MI), ischemia reperfusion and HF [88, 105-107]. For instance, Cao et al., reported that using (S)-2-(11-(nonyloxy) undec-8(Z)-enamido) succinic acid (NUDSA), an EET agonist, in a murine model of MI is associated with improved systolic dysfunction, decreased myocardial fibrosis and limited remodeling in post-infarcted HF [108]. Similarly, administration of an orally active EET mimetic in hypertensive rats exposed to ischemia-reperfusion induced cardiac injury, reduced cardiac associated mortality, provided better cardiac function, reduced pulmonary edema, reduced myocardial fibrosis and decreased macrophage infiltration. Moreover, EET mimetic treatment increased the activity of hem-oxygenase 1 (HO-1) following ischemia-reperfusion injury and attenuated the progression of HF [109]. In a murine model of HF, treatment with sEH inhibitor, adamantan-3-(5-(2-(2-ethylethoxy) ethoxy) pentyl) urea (AEPU) to limit epoxylipid metabolism, prevented pressure overload induced cardiac hypertrophy and decreased susceptibility to ventricular arrhythmias. Furthermore, treatment with AEPU decreased the translocation of NF-κB from the cytosol into the

nucleus in mouse neonatal cardiomyocytes subjected to Angiotensin II (Ang II)-induced hypertension and hypertrophy[110]. Moreover, transgenic mice with cardiomyocyte overexpression of CYP2J2 subjected to pressure-overload or long term infusion of isoproterenol demonstrated reduced hypertrophy and arrhythmogenic events [111]. Evidence suggest that EETs play a major cardioprotective role by decreasing secretion of pro-fibrotic factors leading to reduced remodeling [112]. Mice with cardiac overexpression of CYP2J2 showed improved cardiac function, decreased myocardial hypertrophy and fibrosis resulting in amelioration of cardiac remodeling. Further defining the cardioprotective role of CYP2J2, neonatal cardiomyocytes from mice with cardiac overexpression of CYP2J2 showed decreased level of cardiac remodelling proteins such as collagen type I, and TGF-β in response to Ang II compared to their WT counterparts [112]. Although the exact mechanism of cardioprotective effects of EETs remains unknown, these studies established evidence that EETs have promising therapeutic effects for improving cardiac outcomes.

The evidence suggesting N-3 PUFAs have cardioprotective effects against pathological conditions is controversial [88]. Experimental animal studies suggest there are beneficial effects of N-3 PUFAs toward CVD. For example, in a mouse model of HF, increased myocardial EPA and DHA levels following dietary supplementation attenuated left ventricular (LV) chamber dilation against pressure-overload induced cardiomyopathy providing a proof-of-concept. Moreover, following TAC, mice fed with EPA and DHA showed improved mitochondrial function documented by preservation of citrate synthase and medium chain acyl-CoA dehydrogenase (MCAD) activity [113]. However, differences in clinical literature from several prospective observational studies and large-

scale clinical trials testing the protective effects of N-3 PUFAs have had mixed results [87]. The "GISSI-HF" trial demonstrated N-3 PUFA supplementation was associated with reduced HF-related hospital admissions and mortality in patients with reduced ejection fraction [114]. Conversely, a randomized double-blind trial by the "Alpha-Omega Trial Group" concluded there was no significant benefit for N-3 PUFA toward cardiovascular events post-MI [115]. These differences may be partially attributed to study design, such as inclusion of populations with a high baseline intake of N-3 PUFA and differing doses of EPA and DHA, yet none investigates the role of the CYP-derived epoxy metabolites.

Both preclinical and clinical studies have furnished a wealth of evidence in support of cardioprotective effects of epoxy fatty acids [88, 107, 113, 116]. However, their short half-life limit their therapeutic use and clinical application requiring new strategies to improve their pharmacokinetics [88, 117]. The gene encoding sEH, Ephx2, is the primary enzyme metabolizing PUFA epoxides resulting in the formation of respective diol metabolites via the addition of a water molecule [182, 183]. Novel pharmacological approaches that selectively inhibit sEH, have evolved as clinical tools in various cardiovascular diseases, including hypertension, cerebral ischemia, cardiac ischemia, cardiac hypertrophy, myocardial infarction, and atherosclerosis [118-123]. Pharmacological sEH inhibition is demonstrated to improve both LV diastolic and systolic function and attenuate myocardial remodeling in established HF [105, 124, 125]. Treatment with cis-4-[4-(3-adamantan-1-yl-ureido) cyclohexyloxy] benzoic acid (c-AUCB) attenuated increased systolic and diastolic LV cavity diameter following ischemia/reperfusion injury in rats. Interestingly, the combined administration of EET-mimetic and c-AUC amplified the cardioprotective effects of the single therapy and

significantly decreased MI- induced chamber dilation accompanied with improved systolic function [105]. Chronic inhibition of sEH substantially attenuated lung congestion and albuminuria parallel to preserved cardiac function and structure, acknowledging sEH as a therapeutic target for the treatment of cardiac dysfunction associated with chronic kidney disease [126, 127]. Despite considerable research on the role of sEH, significant gaps remain at many levels in the understanding of mechanisms involved in beneficial effects of sEH inhibition in the setting of cardiovascular diseases.

CYP-dependent oxidation of LA results in the production of epoxyoctadecanoic acids (EpOME), which are rapidly metabolized by sEH to their corresponding diol metabolites, dihydroxyoctadecanoic acids (DiHOME) [88]. Evidence from animal and in vitro studies has suggested DiHOMEs are potent cytotoxic metabolites [128]. Increased levels of cardiac DiHOMEs are thought to be associated with deteriorated myocardial electrical activity, altered ion channel kinetics, depressed LV function and impaired mitochondrial respiration [129-132]. The cardiotoxic effects of DiHOMEs remained evident when cardiac specific over-expression of CYP2J2 failed to improve cardiac functional recovery following ischemia reperfusion, attributed to age-related accumulation of DiHOMEs in the heart [133]. Indeed, the deleterious myocardial effects of DiHOMEs counter the cardioprotective effects of increased epoxylipids [134]. These data suggest that cardioprotection of sEH inhibition could be mediated, at least in part, by inhibiting the production of cardiotoxic DiHOMEs. However, further investigation into cardiac effects of DiHOMEs is warranted.

1.8 Mitochondria: effects of N-3 and N-6 PUFA derived epoxylipids

Mitochondria play a fundamental role in cardiac aging by regulating a plethora of age-associated cardiac changes [135]. Therefore, targeting mitochondrial dysfunction in aging myocardium is an unmet need holding a significant promise for age-related cardiac diseases. Although the exact mechanisms of how epoxylipids regulate cardiac function are not fully understood, accumulating data suggest mitochondria-targeted effects are an important component of their cardioprotective properties [119, 128, 136]. Numerous in vivo and ex vivo studies demonstrate sEH inhibition or treatment with epoxylipids improve LV functional recovery in murine hearts following ischemia-reperfusion injury protecting mitochondrial function and ultrastructure [92, 122, 136-140]. Both genetic deletion of sEH and cardiac overexpression of CYP2J2 limited mitochondrial MPTP opening and preserved mitochondrial ultrastructure in mouse hearts following ischemia-reperfusion injury [92]. Previously, we reported hearts perfused with UA-8 (13-(3-propylureido) tridec-8-enoic acid) a synthetic dual-action compound possessing EET mimetic and sEH inhibitory properties, improved post-ischemic contractile function and reduced infarct size following ischemia-reperfusion injury. These cardioprotective effects were attributed to the ability of UA-8 to prevent the collapse of mitochondrial function and limit the loss of mitochondrial membrane potential, resulting in preserved heart function [141]. Inhibition of endogenous EET production by a selective epoxygenase inhibitor, MS-PPOH, resulted in disruption of mitochondrial ATP generation, increased

ROS production, mitochondrial depolarization and mitochondrial fragmentation in cultured neonatal hippocampal astrocytes. [142]. Both treatment with exogenous EET and CYP2J2 overexpression suppressed ROS production, and increased expression of catalase, as well as cytosolic and mitochondrial superoxide dismutase leading to improved viability in human pulmonary artery endothelial cells subjected to anoxia/reoxygenation [143]. Administration of an EET agonist effectively ameliorated obesity-induced cardiomyopathy by improving mitochondrial function and energy metabolism in cardiac tissues leading to enhanced tolerance to glucose challenge, associated with increased cardiac expression of PGC-1α, a key regulator in mitochondrial biogenesis [144]. Moreover, in the same study, EET-treated mice showed increased level of MnSOD and Mfn-2 in adipose tissues, contributing to balancing the mitochondrial function and redox status [144]. Ablation of sEH gene in mice decreased degradation of EETs leading to a significant increase in Mfn-1, HO-1 and cytochrome c oxidase subunit I (COX-I) level in adipose tissue compared to WT control mice, further revealing a key role for EETs in regulating mitochondrial integrity and function [145]. sEH deletion, also maintained both mitochondrial respiratory ratio (RCR) and ATP generation in isolated murine cardiac fibers subjected to ligation of left anterior descending artery (LAD) in both young and aged mice, contributing to sustained systolic and diastolic function. Moreover, both young and aged sEH null mice demonstrated an improved mitochondrial ultrastructure following MI characterized by improved cristae density and organization [122].

Although the effects of N-3 PUFAs on mitochondria are less extensively studied and characterized, they are increasingly recognized to protect the heart by preserving mitochondrial function [146, 147]. A DHA-rich diet significantly improved ROS-induced

MPTP opening in interfibrillar mitochondria and decreased mitochondrial membrane viscosity associated with a modest attenuation of LV dysfunction in rats with HF [148]. Both EPA and DHA exhibited an up-regulatory effect on expression of Mfn-2 resulting in a significant recovery of mitochondrial network architecture and morphology accompanied by increased ATP production in steatotic HepG2 cells incubated with oleate and palmitate [149]. Anti-apoptotic and pro-survival effects of n-3 PUFAs by shifting the cell death pathway toward survival have been also reported [94, 143]. DHA attenuated apoptosis evidenced by increased Bcl-2 and decreased Bax and cleaved caspase-3 via upregulating OPA-1 and ameliorating mitochondrial fragmentation following subarachnoid hemorrhage in rats subjected to ligation of the carotid artery [150].

Sirt-3 is a nicotinamide adenine dinucleotide (NAD) dependent histone deacetylase found predominately in mitochondria which has been identified as a key mediator in age-related cardiovascular physiology, regulating mitochondrial oxidative stress via deacetylating MnSOD [151, 152]. Cardiomyocytes lacking Sirt-3 show age-dependent mitochondrial swelling and accelerated signs of cardiac aging, including myocardial hypertrophy and accumulated fibrotic tissue [69]. Interestingly, while the cardiac expression of sEH is significantly increased in cardiac aging, genetic deletion of sEH attenuated the age-related decrease in Sirt-3 activity in female mice [153]. The effect was associated with higher levels of active mitochondrial MnSOD resulting in better overall cardiac function suggesting the preservation mitochondrial integrity in aged mice [153]. This data fosters an important body of research on the underlying mechanisms involved in effects of epoxylipids on mitochondrial redox apparatus in cardiac aging and age-related pathogenesis.

Dysregulation of mitochondrial dynamics and mitophagy found in aged hearts is associated with an accumulation of damaged mitochondria and subsequent cardiac dysfunction [52]. Suppression of sEH demonstrated to increase expression of Mfn-1 associated with improved cardiac mitochondrial function and biogenesis, increased ATP production, ameliorated cardiac inflammation and consequently protect the heart from metabolic syndrome.[154]. EETs also maintained increased expression of Mfn-2 and MnSOD in obesity-induced cardiomyopathy shedding more light on their role in maintaining mitochondrial homeostasis [144]. While the mechanisms remain unknown there appears to be a role for epoxylipids in regulating mitochondrial function in aged hearts.

1.7 Sex differences and N-3 and N-6 polyunsaturated fatty acids

Biological aging is associated with slowly progressive deterioration in cardiac function and structure predisposing elderly adults to cardiovascular diseases [155]. There is growing evidence that significant sex differences exist in presentation, progression and treatment responses as individuals age, resulting in different clinical outcomes between men and women highlighting the importance and necessity for considering sex differences in aging studies [156-158]. Males present greater LV mass and chamber dimensions with increased susceptibility toward developing eccentric LV remodeling, systolic dysfunction and DCM at younger ages [159]. However, upon aging, increased myocardial wall thickness has been reported to occur in females, which may be accompanied by concentric LV chamber remodeling and diastolic dysfunction, predisposing females to HF [156, 158]. Furthermore, there are several cardiovascular risk factors exclusively experienced by

women, including menopause, early menarche, preeclampsia, and pregnancy [160]. The higher incidence of CVD in men compared to women prior to menopause suggests sex hormones play key role in development [161]. However, emerging research has indicated sex hormones alone, are insufficient to fully explain the variations in cardiac outcomes between men and women [162]. Interestingly, the data pertaining to biological sex-differences show distinct disparities in male and female mitochondrial function and morphology [163]. Female mitochondria are more differentiated with increased cristae density and protein content resulting in lower levels of free radical production and higher efficiency [163-165]. In view of the fact that mitochondria are strongly associated with age-related cardiac differences, comparable mitochondrial sex-differences appear to be a promising theory to explain sex-specific differences in cardiac aging. Sexual dimorphism has been documented in protein expression and regulation of sEH activity in both cardiac and extra-cardiac tissues [153, 166-168]. While sEH inhibition did not have discernable effects on female mouse systolic blood pressure (SBP), genetic deletion of sEH efficiently normalized SBP in male mice [168]. Sexual disparity in regulating sEH activity becomes more pronounced in aged tissues, as aging is associated with a significant increase sEH in male animals raises new questions on the mechanisms associated with sex-specific responses to sEH-based interventions [153, 166].

1.8 Thesis overview

1.8.1 Rationale

CVD remains the leading cause of mortality in both men and women of all ages worldwide [169-171]. Aging is a key determinant of cardiovascular health, as evidenced from an exponential increase in the prevalence of CVDs in the geriatric population [1]. Yet, our understanding of the progression of cardiac aging is largely exclusive to a collection of intriguing yet unconnected data sets bridging the age-associated cardiac alterations and age-related CVD. Identifying novel approaches to modulate aged-induced cardiac dysfunction will provide insights into development of effective therapeutics with limited adverse effects.

Epoxylipids, endogenous metabolites of N-3 and N-6 PUFAs, have been demonstrated to elicit cardioprotection against various cardiac injuries via their anti-inflammatory, vasodilatory, anti-fibrinolytic, and anti-apoptotic properties, as well as protective effects on mitochondria [88, 139]. The activities of epoxylipids are regulated through their metabolism by sEH. As such, pharmacologically inhibiting or genetic deletion of sEH to increase epoxylipids bioavailability can be regarded as a therapeutic target for CVDs [172]. Several mechanisms contribute to cardioprotective effects of epoxylipids, from which, maintaining mitochondrial structure and function is of great importance in the platform of aging and age-related CVDs. While our understanding of the mechanisms involved in cardioprotective effects of epoxylipids remains limited, several findings have highlighted their diverse cardioprotective activities and provided ideas toward developing novel therapeutic approaches (Figure 2).

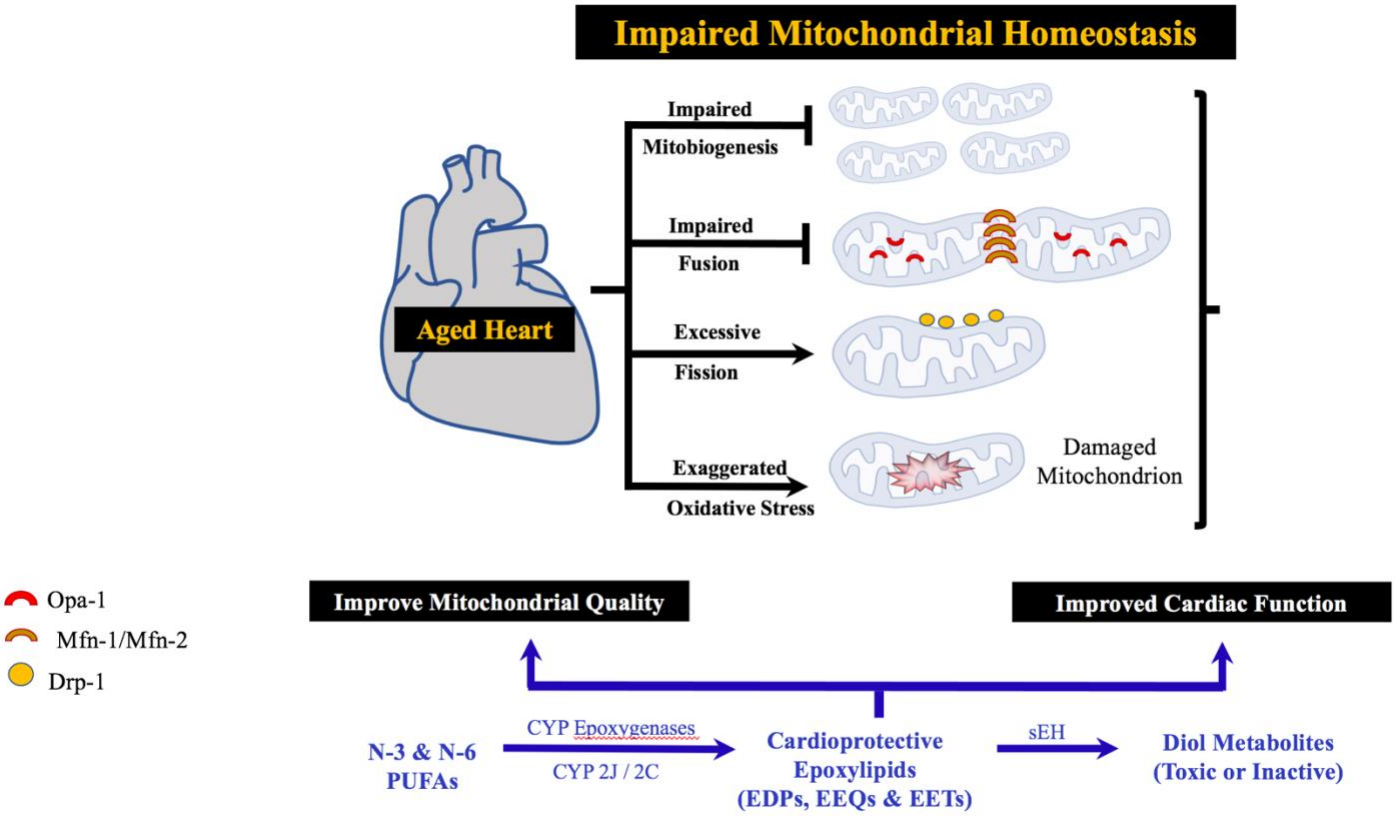

Figure 1.2. Schematic diagram of the potential modulatory effects of CYP-derived epoxylipids against cardiac aging: Cardiac senescence is an intrinsic process accompanied by a general decline in mitochondrial function and impaired mitochondrial homeostasis as evidenced by reduced mitochondrial biogenesis, dysregulated fusion, exaggerated fission and oxidative stress. N-3 and N-6 PUFAs can be metabolized by CYP isoenzymes to their corresponding epoxylipids, which maintain/improve mitochondrial integrity and function, suggesting a proof-of-concept for beneficial effects against age-related cardiac pathologies. DRP-1: dynamin-related protein 1, EET: epoxyeicosatrienoic acid, EDP: epoxydocosapentaenoic acid, EEQ: epoxyeicosatetraenoic acid, IL: interleukin, MFN: mitofusin, OPA-1: optic atrophy 1, PUFA: poly unsaturated fatty acid, sEH: soluble epoxide hydrolase.

1.8.2 Hypothesis

Preliminary data from our lab has provided evidence demonstrating that inhibiting metabolism of epoxilipids by sEH both genetically and pharmacologically improves survival in aged females following myocardial infarction. However, the exact mechanism(s) involved in sex-dependent differences and effects are unknown. Mitochondrial abnormalities are hallmarks of cardiac aging accompanied by decrease in number of myocytes, increase in the size of cardiomyocytes, and over accumulation of lipids and fibrotic areas [173, 174]. A large body of evidence in literature has reported the sex related differences in mitochondria including anti-oxidant properties and level of ROS [175-178]. Based upon evidence from our experimental work demonstrating that epoxylipids protect the cardiac mitochondria and heart function following injury, we hypothesize that CYP P450 epoxygenase derived epoxylipids, initiate a cardioprotective response against age-induced mitochondrial damage.

1.8.3 Thesis aims

1. To investigate age- and sex-related cardiac alterations in murine hearts.

2. To investigate if genetic deletion of sEH confers cardioprotective effects against age-related cardiac alterations.

3. To address sex-specific differences in cardioprotective effects of sEH deletion.

4. To assess the role of sEH inhibition in maintaining mitochondrial structure and function in the aging heart.

Chapter 2: Age and sex differences in hearts of soluble epoxide hydrolase null mice

This chapter includes content previously published in the following research paper:

K Lockhart Jamieson, Hedieh Keshavarz-Bahaghighat, Ahmed M Darwesh, Deanna K Sosnowski, John M Seubert; Age and Sex Differences in Hearts of Soluble Epoxide Hydrolase Null Mice. Front physiol, 2020 Feb 7;11:48.

Abstract

Biological aging is an inevitable part of life that has intrigued individuals for millennia. The progressive decline in biological systems impacts cardiac function and increases vulnerability to stress contributing to morbidity and mortality in aged individuals. Yet, our understanding of the molecular, biochemical and physiological mechanisms of aging as well as sex differences is limited. There is growing evidence indicating CYP450 epoxygenase-mediated metabolites of n-3 and n-6 polyunsaturated fatty acids are active lipid mediators regulating cardiac homeostasis. These epoxy metabolites are rapidly hydrolyzed and inactivated by the soluble epoxide hydrolase (sEH). The current study characterized cardiac function in young and aged sEH null mice compared to the corresponding wild-type (WT) mice. All aged mice had significantly increased cardiac hypertrophy, except in aged female sEH null mice. Assessment of cardiac mitochondria demonstrated an increased expression of acetyl Mn-SOD levels that correlated with decreased Sirt-3 activity in aged WT males and females. Conversely, aged sEH null mice had preserved Sirt-3 activity and better mitochondrial ultrastructure compared to WT mice. Consistent with these changes, the activity level of SOD significantly decreased in WT animals but was preserved in aged sEH null animals. Together, these data highlight novel cardiac phenotypes from soluble epoxide hydrolase null mice demonstrating a sexual dimorphic pattern of aging in the heart.

2.1 Introduction

The prevalence of cardiovascular disease (CVD) has markedly increased as the global population ages [179, 180]. Important age-associated changes resulting in structural deterioration and progressive decline in cardiac function is characterized with development of left ventricular hypertrophy, systolic and diastolic dysfunction and decreased exercise capacity [181]. Although the influence of age on the heart is well-documented, the sex-specific patterns of cardiac aging in males and females are less appreciated [156]. Sex-associated differences, such as a higher incidence of obstructive diseases in males compared to microvascular complications in females, contribute to the variations in cardiac outcomes persistently observed between men and women [182, 183]. Much of the early work into these sex-associated cardiovascular outcomes focused on the role of endogenous hormones as mediators of cardiovascular protection [162]. Recent data suggest that hormonal changes alone are insufficient to fully explain these variations, and other involved biological mechanisms remain a subject of ongoing debate [184].

Mitochondrial dysfunction and increased oxidative stress have been identified as key participants in cardiac aging and associated CVD [185]. Mitochondria are dynamic organelles constantly undergoing fission and fusion events in response to energy demand and cellular stress. The balanced fission and fusion events under basal conditions is responsible for maintaining mitochondrial morphology and metabolism [17]. Key proteins regulating fission include dynamin related protein 1 (Drp-1), while mitofusin 1 and 2 (Mfn-1 and 2) are involved in mitochondrial fusion in mammals. Altered expression or activation of mitochondrial dynamic proteins have been implicated in the pathogenesis of cardiac diseases [9]. Evidence is suggesting the imbalance between mitochondrial fission

and fusion during aging has a role in age-related CVD via compromising mitochondrial integrity [8, 51, 52].

Based on an oxidative stress hypothesis in aging, both increased levels of ROS and a decline in efficiency of antioxidant systems contribute to age-associated progressive degeneration in cardiac function and structure [40]. Thioredoxin interacting protein (Txnip) has been identified as a tumor suppressor protein with a primary role of inhibiting antioxidant activity of mitochondrial thioredoxin system (Trx2) via direct interaction [42]. Age-dependent upregulation of Txnip leads to accumulation of ROS, increased oxidative stress and perturbation of cellular redox equilibrium [44]. Increased levels of Txnip have been documented in the process of aging and age-related diseases contributing to unbalanced oxidative stress, a hallmark of aging [41, 44, 45].

Sirtuin 3 (Sirt-3) is a deacetylase enzyme primarily localized in the mitochondria involved in regulating several physiological and pathophysiological processes, including mitochondrial dynamics and redox homeostasis, through the deacetylation and activation of various proteins [186, 187]. Sirt-3 increases the expression of Mfn-2, thereby slowing the excessive mitochondrial fusion caused by abnormal Drp-1 [188]. Moreover, Sirt-3 directly activates the major mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD), which scavenges reactive oxygen species (ROS) [75]. Evidence has shown Sirt-3 may be down regulated during cardiac aging resulting in suppressed MnSOD activity leading to increased ROS levels [71]. Subsequently, increased ROS levels can activate downstream targets, including the PI3K/Akt pathway, further exacerbating age-related cardiac hypertrophic response [71, 189, 190]. Understanding the exact role Sirt-3

has in the aging processes remains a focus of many research groups trying to uncover key pathways and therapeutic approaches to treat age-related complications [69, 79, 191, 192].

Polyunsaturated fatty acids (PUFAs) are metabolized through numerous metabolic pathways, including the cyclooxygenase, lipooxygenase and cytochrome P450 (CYP) monooxygenase pathways [88]. These transformations produce a plethora of lipid mediators with numerous biological functions [118, 119, 193]. Oxidative metabolism of PUFAs can produce bioactive mediators, termed oxylipids, which are further metabolized to less bioactive diols by the epoxide hydrolase family of enzymes (EH) [194, 195]. Located primarily in the cytosol, the soluble form (sEH), has been implicated in the progression of multiple cardiovascular diseases, including hypertension and atherosclerosis [196]. The microsomal form (mEH) is also an established xenobiotic-metabolizing enzyme responsible for the biotransformation of active metabolites [197]. While mEH is capable of hydrolyzing PUFA derivatives, it has been determined to have limited roles in cardiac metabolism[198, 199]. Cardiac sEH primarily metabolizes oxylipid mediators to less active metabolites, which often results in loss of cardioprotective properties [200]. Both genetic deletion and pharmacological inhibition of sEH has been demonstrated to mediate cardioprotective, anti-inflammatory and anti-hypertensive responses, as well limit mitochondrial injury [88, 122]. In humans, genetic polymorphisms increasing sEH activity are associated with poor outcomes in cardiac and renal disease, although this seems to be population-dependent [201-203]. Numerous animal studies have demonstrated the importance of sEH in various models of CVD; however, there is limited information regarding its role in generalized cardiac aging [119, 122, 204-206]. Moreover, there is limited information regarding sexual disparity in

cardiac sEH with age [166, 168], as such the present study investigated the impact of sEH in age- and sex-dependent cardiac differences.

2.2 Material and methods

2.2.1 Animals

A colony of mice with targeted deletion of the Ephx2 gene (sEH null) with their WT littermates are maintained at the University of Alberta. Mice are conserved on a C57BL6 background. All experiments were carried out on male and female mice aged 2-4 months old (young) and 15-18 months old (middle-aged). The middle-age range, referred to henceforth as "aged", was chosen to be clinically representative of the manifestation of cardiovascular disease in humans and to avoid confounding effects of frailty, which can drastically change cardiovascular phenotypes in elderly mice [207]. At the appropriate age, hearts were excised from mice following euthanasia with 100mg/kg of sodium pentobarbital. Hearts and kidneys were then rinsed in 1X PBS, flash frozen in liquid nitrogen and stored at -80ºC awaiting analysis. Animal experimental protocols were approved by the University of Alberta Health Sciences Welfare Committee and were in carried out in accordance with the guidelines set by the Canadian Council of Animal Care.

2.2.2 Protein expression and immunoblot analysis

Western blot analysis was used to determine protein expression in subcellular mitochondrial, microsomal, and cytosolic fractions. Briefly, hearts and kidneys were harvested, ground into finely powders and homogenized in approximately 100-120 µL ice cold homogenization buffer (250mM sucrose, 10mM Tris–HCL, 1mM EDTA, 1mM sodium orthovanadate, 1 mM sodium fluoride, 10µg/L aproptinin, 2µg/L leupeptin and 100µg/L pepstatin) and centrifuged at 700 x g for 10 min. The supernatant was then

centrifuged at 10 000 x g for 20 minutes and the subsequent pellet containing mitochondria was resuspended in 70 μL homogenization buffer. The resultant supernatant was centrifuged at 100, 000 x g for 60 min with the supernatant taken as the cytosolic fraction and the pellet taken and resuspended as the microsomal fraction. Protein levels were quantified in subcellular fractions using standard Bradford assay. Samples containing 35μg protein were loaded on 4-15% TGX® gels (BioRad, CAN) and used for SDS-PAGE gel electrophoresis, then transferred onto 0.2μm PVDF membranes for subsequent western blotting. Probing was done using primary antibodies against sEH (1:500, Elabscience; E-AB-60489), total-Akt (1:1000, Cell Signaling; CS9272S), ser473 phospho-Akt (1:1000, Cell Signaling; CS5106S), Drp-1 (1:100, Cell Signaling; CS8570S), Mfn-2 (1:1000, Cell Signaling; CS9482S), Sirt-3 (1:1000, Cell Signaling; CS5490S), total MnSOD (1:5000, Abcam; ab13533), acetyl-MnSOD (1:5000, Abcam; ab13707), α-tubulin (1:1000, Abcam, ab4074), mEH (1:200, Santa Cruz, sc135984), Txnip (1:1000, Cell Signaling; CS14715S), GAPDH (1:1000, Cell Signaling; CS2118S), and VDAC (1:1000, Abcam; ab14734). After washing with 1X TBST, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:5000) and visualized with ECL reagent. The densitometry analysis was performed based on relative band intensities using Image J software (NIH, USA).

2.2.3 Enzymatic assays

Sirt-3 activity was detected in the isolated mitochondrial fractions using a Sirt-3 fluorescent assay kit (BPS Bioscience, San Diego, CA, USA), according to the

manufacturer's instructions. In this assay, mitochondria were first isolated from the hearts of young and aged male and female WT and sEH null mice. Mitochondria fractions were mixed with the specific HDAC fluorogenic substrate, bovine serum albumin, NAD+ and assay buffer. The deacetylation process induced by Sirt-3 in the sample sensitizes the HDAC substrate so that subsequent treatment with the Sirt-3 assay developer produces a fluorescence product that was measured using a fluorescence plate reader at 350/460 nm excitation/emission wavelengths. The activity of Sirt-3 was expressed as U/μg protein [208, 209].

As an established biomarker of mitochondrial content, citrate synthase activity was measured spectrophotometrically as previously described [119]. Briefly, heart tissues were ground and homogenized in ice-cold homogenization buffer (20mM Tris, 40mM KCl, 2mM EGTA, pH7.4, with 50mM sucrose added the day of homogenization) and centrifuged at 600 x g for 10 min. The supernatant was used to assess enzymatic activity spectrophotometrically as described previously [210].

SOD activity was measured in the cytosolic fractions using a spectrophotometry-based assay dependent upon the competition for superoxide anion (O2-) by cytochrome c and SOD. The assay utilized xanthine and xanthine oxidase as the primary source of O2-. In this assay, one unit of SOD is equal to the amount of the enzyme which inhibits 50% of the rate of the reduction of cytochrome c [211, 212].

Protein carbonyl content was assessed in cytosolic fractions based on a reaction with 2,4-dinitrophenylhydrazine derivatization (DNPH) using a protein carbonyl ELISA kit (Abcam; Ab1238536) following manufacturer specifications.

2.2.4 Mitochondrial ultrastructure

Conventional transmission electron microscopy (TEM) was used to assess mitochondrial ultrastructure. A 1-2mm^3 sample of myocardial tissue was obtained mid-level from the left ventricular free wall and fixed at 4°C overnight in 3% glutaraldehyde and 3% paraformaldehyde. A mixture of 1.5% potassium ferrocyanide [K4Fe(CN)] and 2% osmium tetroxide (OsO4) in 0.1M cacodylate buffer was used as a post-fixative followed by staining en bloc with 2% uranyl acetate (pH 5.2) for one hour. Tissues were dehydrated in a continuous series of ethyl alcohol (30, 50, 70, 80, 90, 95, 100%) followed by acetone. Resin infiltration was obtained with serial dilutions of acetone:Spurr's resin (2:1; 1:1; 2:1; absolute Spurrs's resin). The samples were then thermally polymerized for 24h at 70°C, followed by ultra-thin sectioning (70nm thickness) using an ultramicrotome (Leica UC7, Leica Microsystems Inc., Vienna, Austria). Samples were post-stained with 4% uranyl acetate and Reinolds' lead citrate for 30min followed by carbon-coating (Leica EM ACE600, Leica Microsystems Inc., Vienna, Austria). Sections were imaged at 60kV using a transmission electron microscope (Hitachi H-7650 TEM, Hitachi High-Technologies Canada, Inc.) equipped with a 16-megapixel EMCCD camera (XR111, Advanced Microscopy Technique, MA, USA) within one week of post-staining.

2.2.5 Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical significance ($P < 0.05$) was determined by three-way ANOVA with Tukey's post-hoc test. Statistical analysis was performed using GraphPad Prism 8 software.

2.3 Results

2.3.1 Age-related cardiac hypertrophy is prevented in aged sEH null female mice

Significant increases in body weight were observed in all aged mice of both sexes and genotypes (Fig.2.1A). The ratio of heart weight (HW) to tibia length (TL) was used as an index of cardiac hypertrophy. There were no differences in cardiac weights between young WT and sEH null animals of either sex (Fig.2.1B). Both aged male and female WT mice and male sEH null mice demonstrated significant increases in HW:TL; however, no increases were observed in aged female sEH null mice (Fig.2.1B). While the Akt pathway has an important role as a pro-survival pathway, increased activation of Akt over aging has been shown to contribute to age-related cardiac hypertrophy and inflammation [213, 214]. Consistent with the literature, immunoblotting results indicated significantly increased levels of pAkt in the cytosolic fraction in aged mice (Fig.2.2A, B)

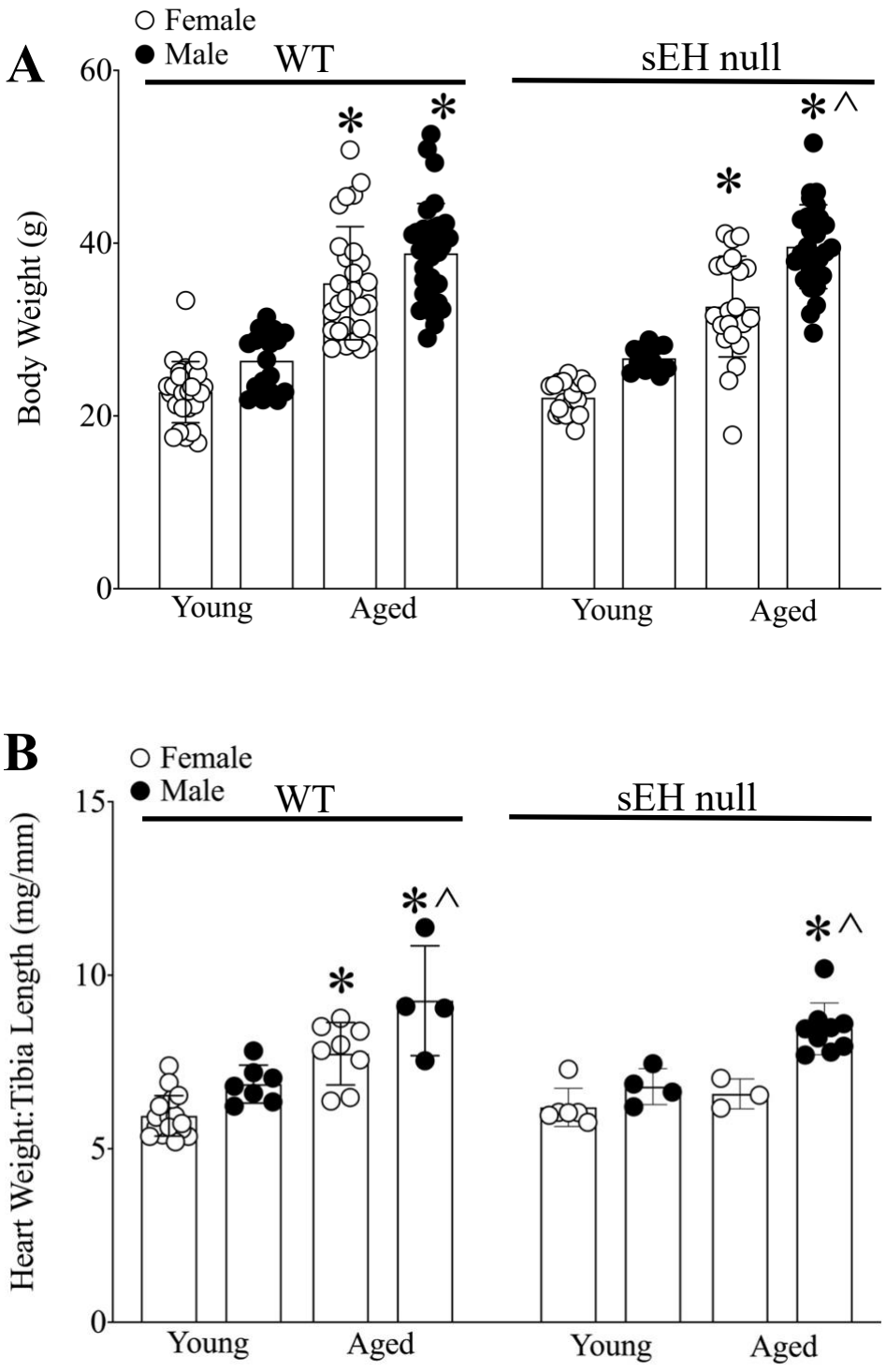

Figure 2.1. Physiological parameters in young and aged WT and sEH null mice: (A) Body weight (g) of mice, (B) heart weight (HW) to tibia length (TL) HW:TL of mice. Values represent mean ± SEM, n=3-15, $P < 0.05$, *vs. young counterpart; ^vs female counterparts.

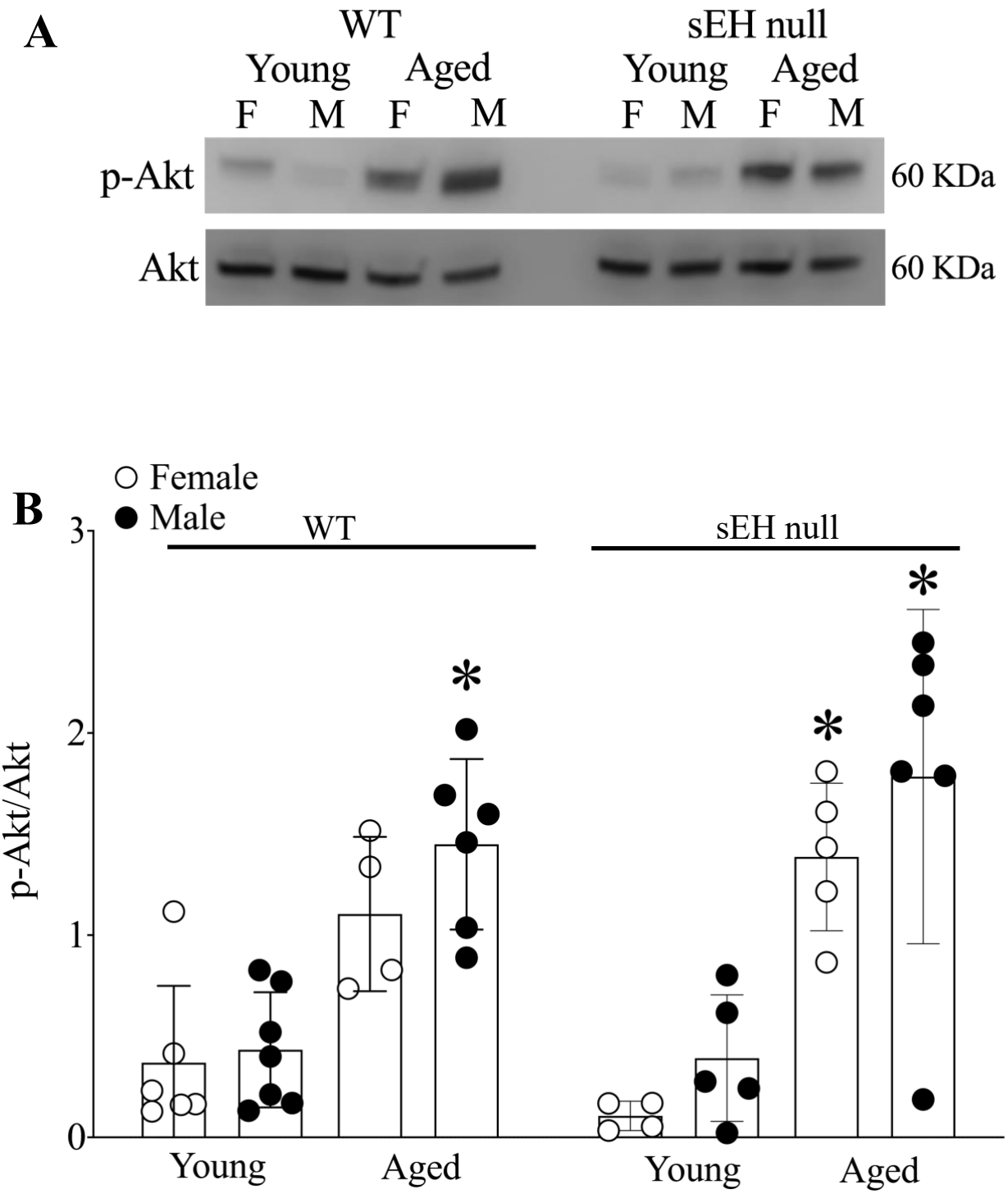

Figure 2.2. Cardiac pAkt level in young and aged WT and sEH null mice: (A) Representative immunoblot of cytosolic phosphorylated Akt and total Akt protein expression, (B) histogram of relative expression of pAkt:Akt in young and aged WT hearts and sEH null hearts. Values represent mean ± SEM, n=5-8, $P < 0.05$, *vs. young

2.3.2 Aging affects the protein expression of epoxide hydrolases

No expression of sEH was detected in either young or aged hearts from null mice confirming genetic deletion (Fig.2.3A). sEH expression was significantly increased in aged male WT mice but not in females (Fig.2.3A, B). Interestingly, our data demonstrated a significant increase in mEH expression in both aged WT and sEH null females, as well as aged WT males (Fig.2.4A, B). However, such increase was not observed in aged sEH null males, who had significantly decreased mEH expression compared to aged WT males (Fig.2.4A, B). Epoxide hydrolases are also involved in renal epoxylipid metabolism [215]. As renal function is related to overall cardiovascular health, we assessed changes in sEH and mEH in kidneys isolated from young and aged mice. There was no sEH seen in sEH null kidneys, supporting the success of the whole-body knock out (Figure 2.5A, B). Correlating with previous literature, renal sEH expression was increased in WT males, although there was no change over aging (Fig.2.5A, B)[216]. Renal mEH was not significantly altered in any group, although the large variation suggests inter-individual changes may be present (Fig.2.6A, B).

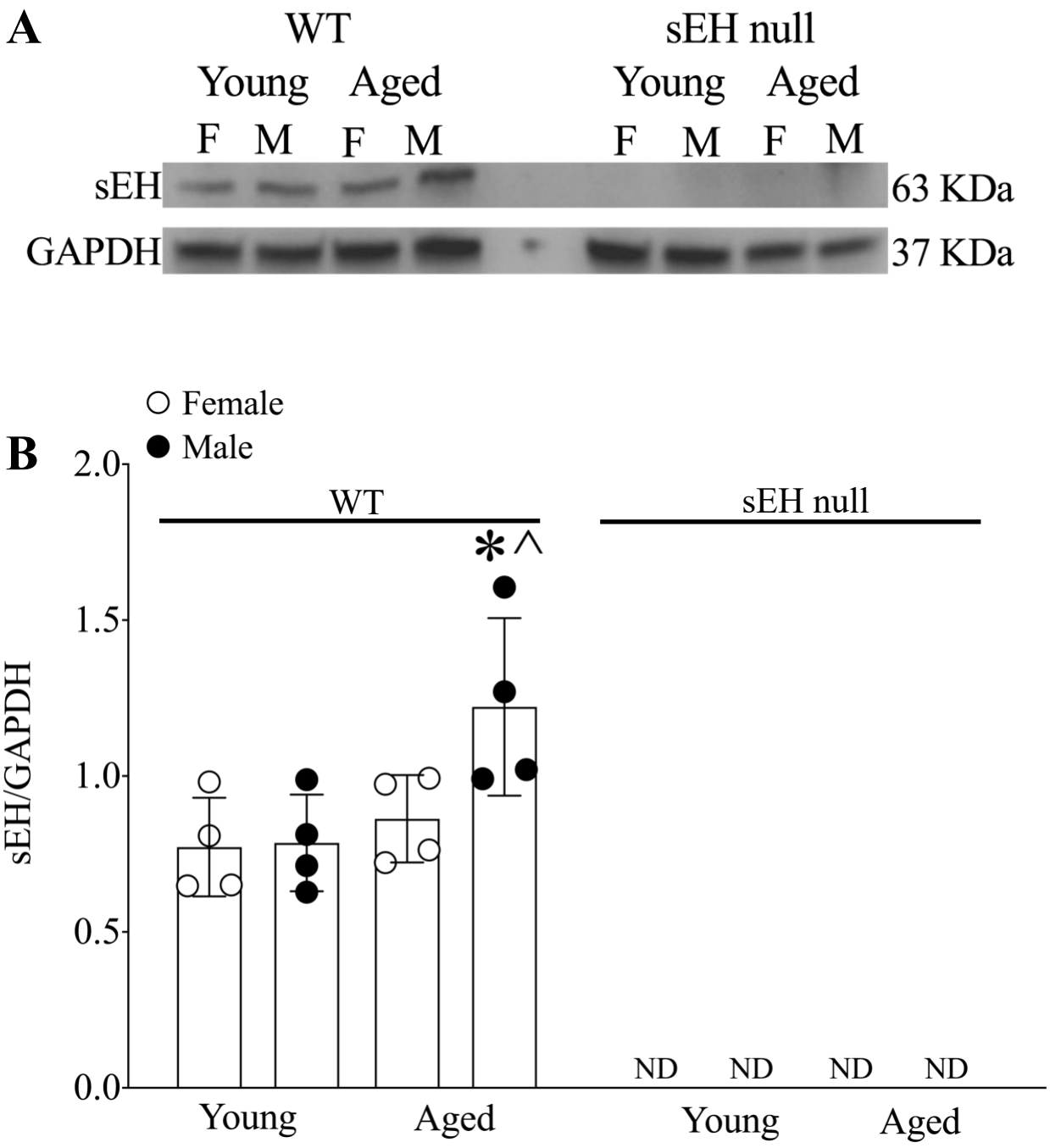

Figure 2.3. Protein expression of cardiac soluble epoxide hydrolases in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac soluble epoxide hydrolase. Protein expression of sEH was normalized to GAPDH. Data represented as mean ± SEM, n=4, P <0.05, *vs. young counterparts; ^vs. female group.

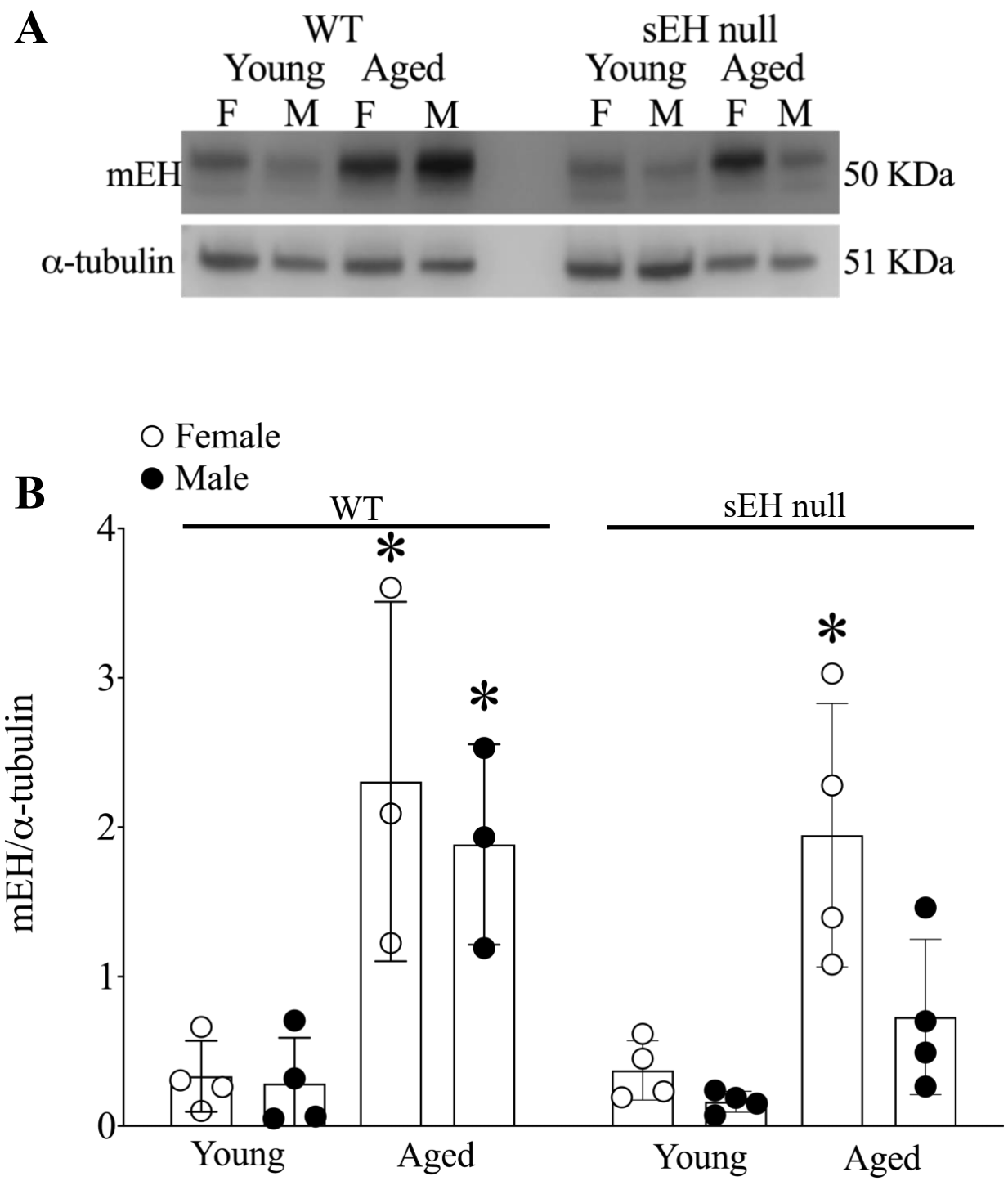

Figure 2.4. Protein expression of cardiac microsomal epoxide hydrolases in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac microsomal epoxide hydrolase. Protein expression of mEH was normalized to α-tubulin. Data represented as mean ± SEM, n=3-5, P <0.05, *vs. young counterparts.

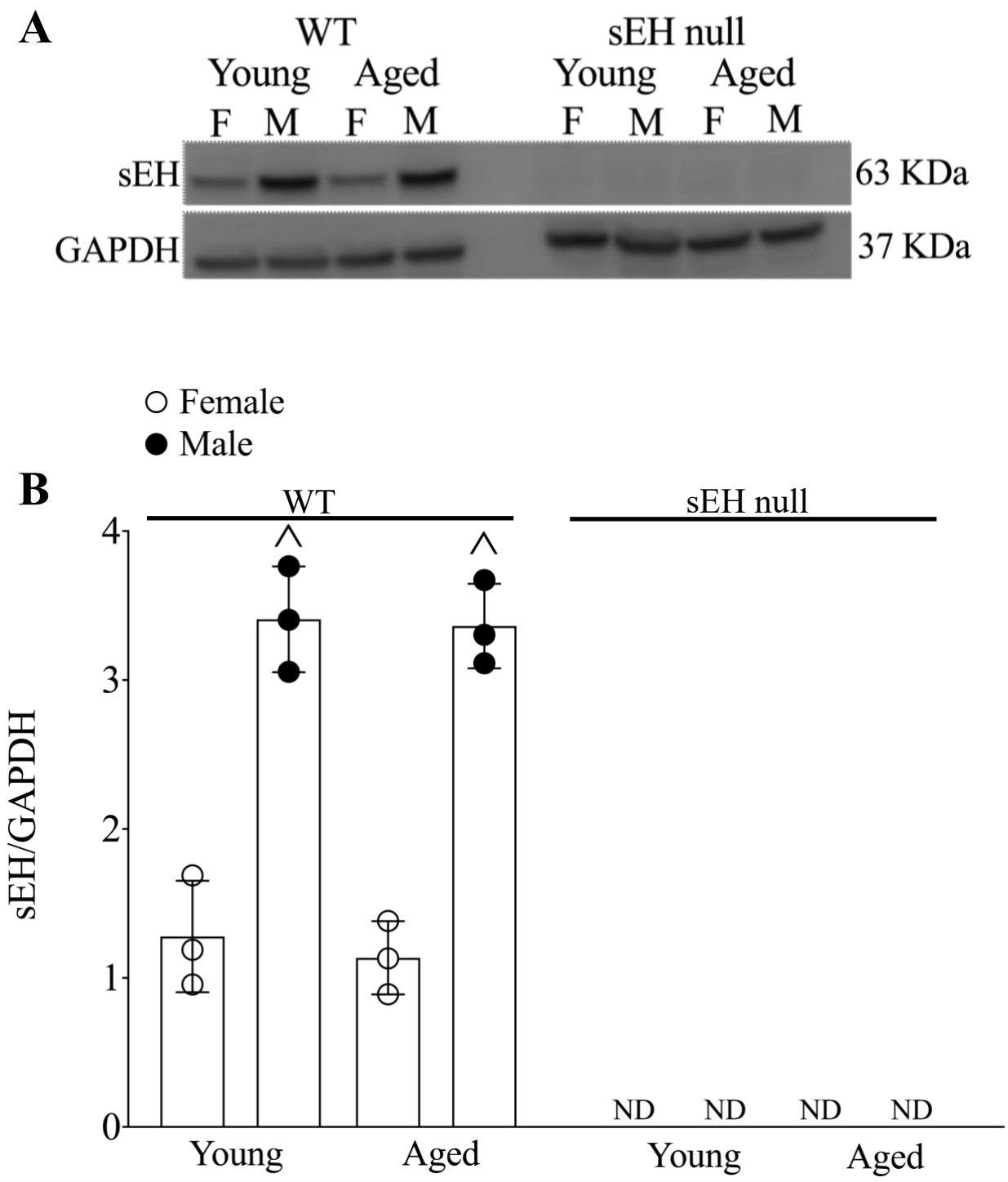

Figure 2.5. Protein expression of renal soluble epoxide hydrolases in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for renal soluble epoxide hydrolase. Protein expression of sEH was normalized to GAPDH. Data represented as mean ± SEM, n=3, P <0.05, ^vs. female group.

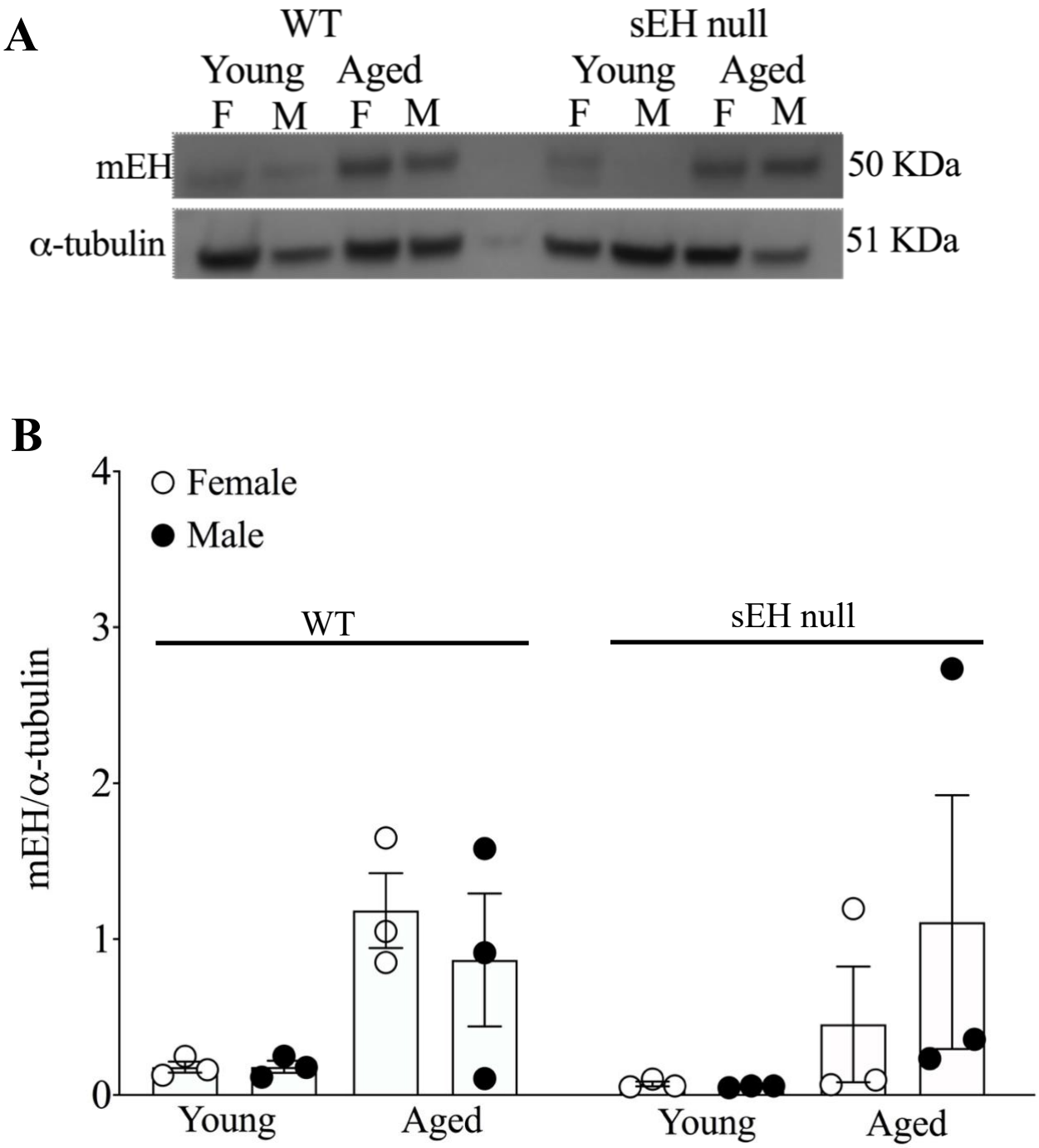

Figure 2.6. Protein expression of renal microsomal epoxide hydrolases in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for renal microsomal epoxide hydrolase. Protein expression of mEH was normalized to α-tubulin. Data represented as mean ± SEM, n=3, P <0.05, *vs. young counterparts.

2.3.3 Markers of oxidative stress; Sirt-3 activity and acetylated MnSOD are preserved in aged sEH null female mice

Sirt-3, the main mitochondrial deacetylase, has been found to be down-regulated with aging and associated with increased ROS levels correlating with a decline in cardiac function [73, 81]. In the current study, there were no differences observed in mitochondrial Sirt-3 protein expression in any group (Fig.2.7A, B). However, Sirt-3 activity was significantly decreased in hearts from aged WT females, with a similar trend in males (P = 0.0726). Interestingly, Sirt-3 activity was preserved in aged sEH null mice compared to their young counterparts and aged sEH null females had significantly higher Sirt-3 activity than similarly aged WT females (Fig.2.8). The aged-dependent changes in Sirt-3 result in reduction in the level of activated MnSOD resulting in increased oxidative stress [214]. Consistent with previous studies, the expression level of AcMnSOD significantly increased in an age-dependent manner in both male and female WT hearts (Fig.2.9A, B). Interestingly, AcMnSOD expression was lower in sEH null mice compared to their WT counterparts (Fig.2.9A, B). Both aged male and female sEH null mice demonstrated increased AcMnSOD levels but these were significantly lower than the corresponding aged WT mice (Fig.2.9A, B). In accordance with literature, cardiac SOD activity was significantly decreased in both male and female WT aged animals (Fig.2.10). SOD activity was preserved in aged sEH null animals compared to the young null mice (Fig.2.10). These data suggest sEH genetic deletion confers protection against oxidative stress via preserving AcMnSOD levels and SOD activity. This was partially supported by assessment of protein carbonylation as a biomarker of oxidative stress [217], where increased levels of protein carbonyl were only observed in aged male animals regardless

of their genotype and not in aged female hearts (Fig.2.11). Protein expression of Txnip, as a key regulator of mitochondrial oxidative stress was also assessed in cytosolic fraction of mouse hearts and did not reveal any significant differences between different groups (Fig.2.12A, B). Renal AcMnSOD and Sirt-3 expression remained unchanged over aging in any group (Fig.2.13A, B; Fig.2.14A, B).

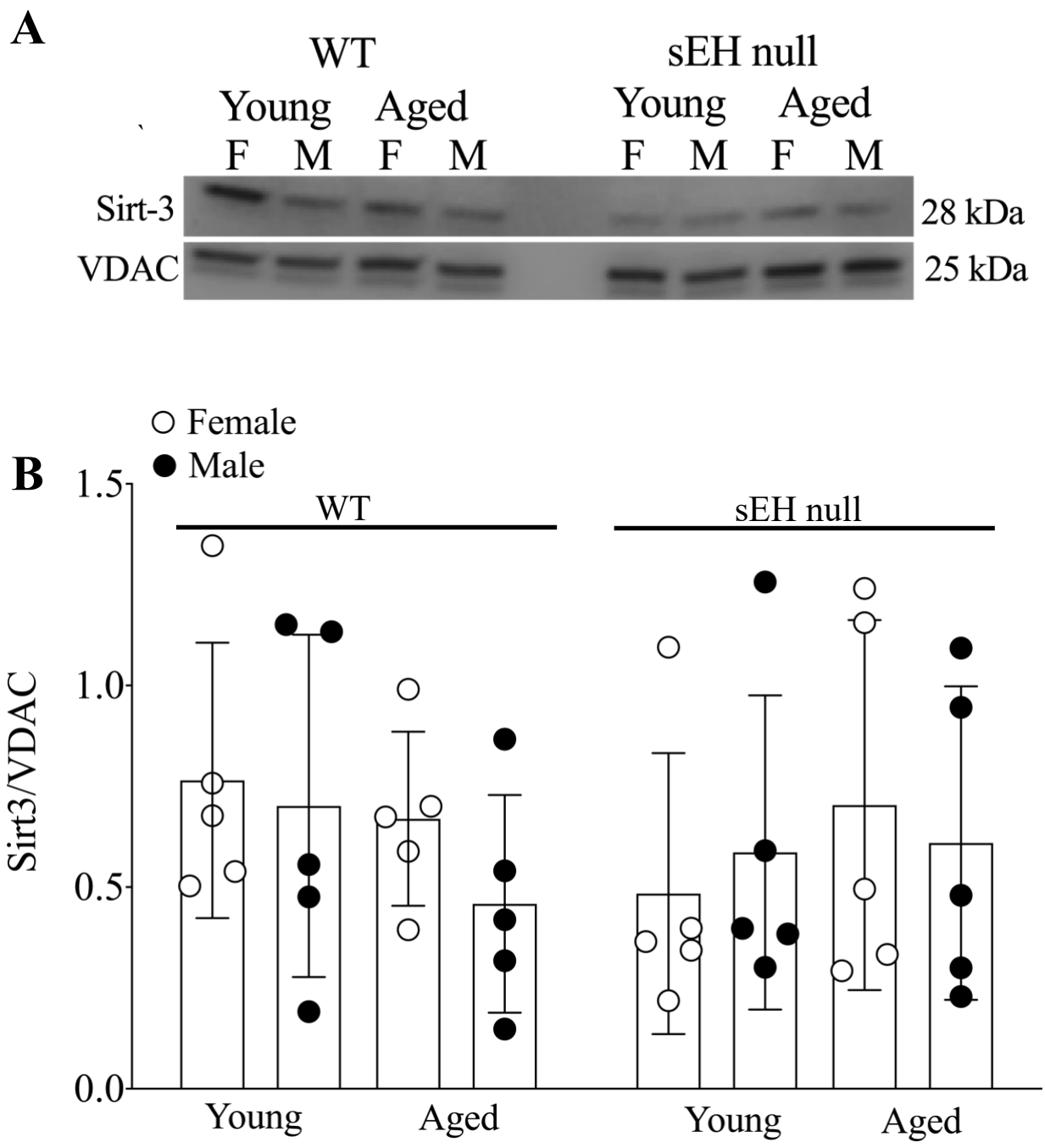

Figure 2.7. Protein expression of cardiac sirtuin-3 in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac sirt-3. Protein expression of Sirt-3 was normalized to VDAC. Data represented as mean ± SEM, n=5, P <0.05.

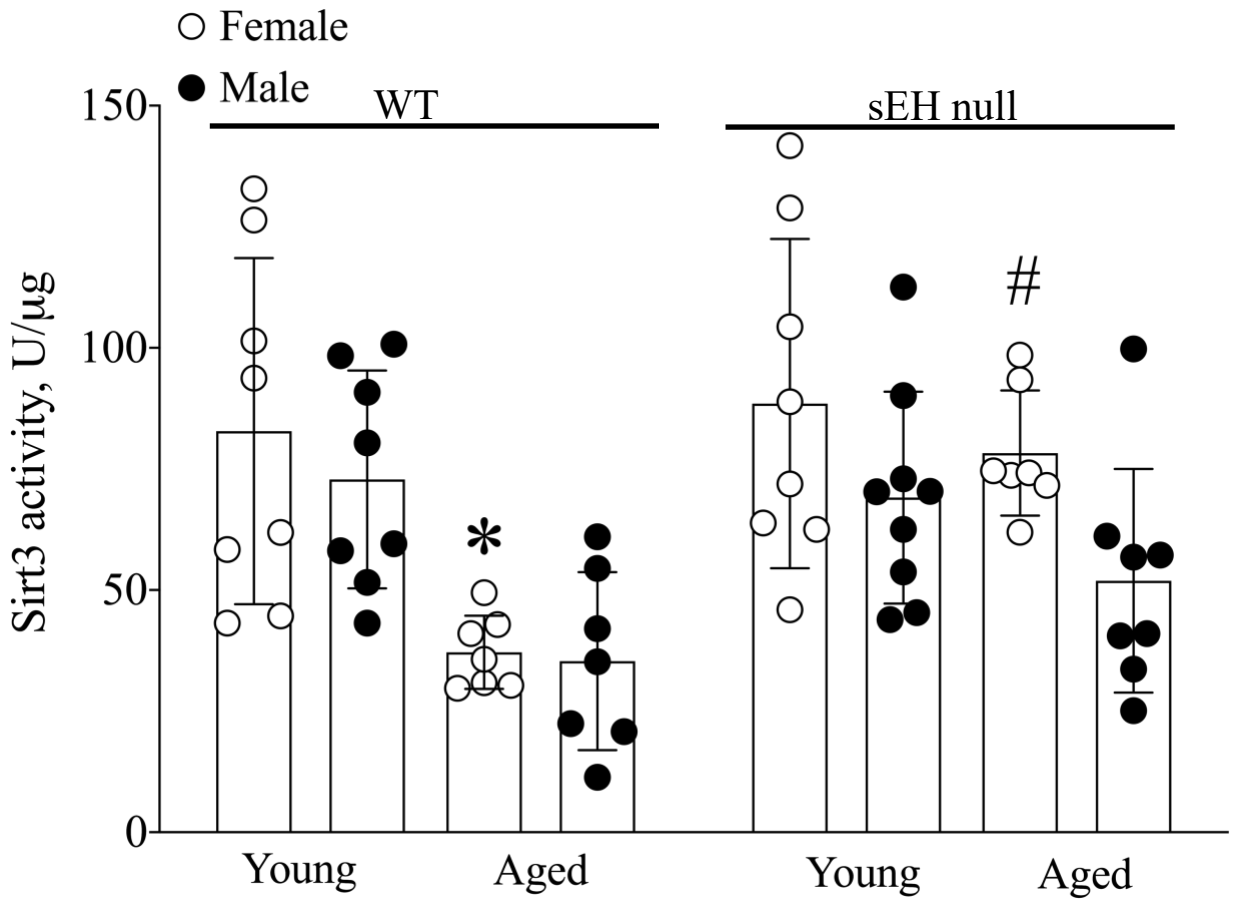

Figure 2.8. Cardiac sirtuin-3 activity in WT and sEH null mice: Sirt-3 activity in young and aged WT and sEH null mice was determined in mitochondrial fractions. Data represented as mean ± SEM, n=8, P <0.05, *vs young counterparts; #vs WT counterparts.

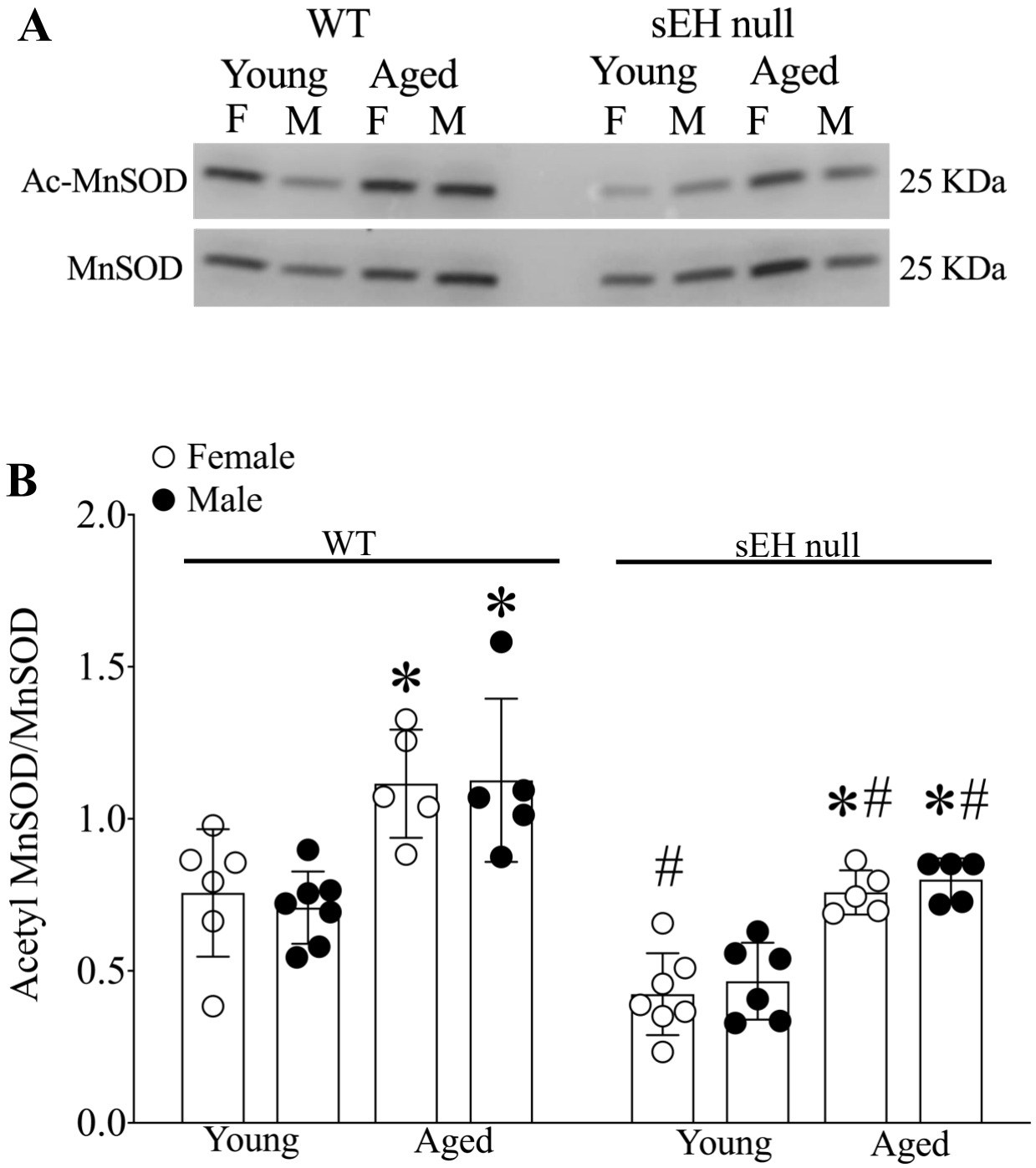

Figure 2.9. Protein expression of cardiac acetyl-MnSOD in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac AcMnSOD. Relative protein expression of AcMnSOD normalized to total MnSOD in young and aged WT hearts and sEH null hearts. Data represented as mean ± SEM, n=5-7, P <0.05, *vs young counterparts; #vs WT counterparts.

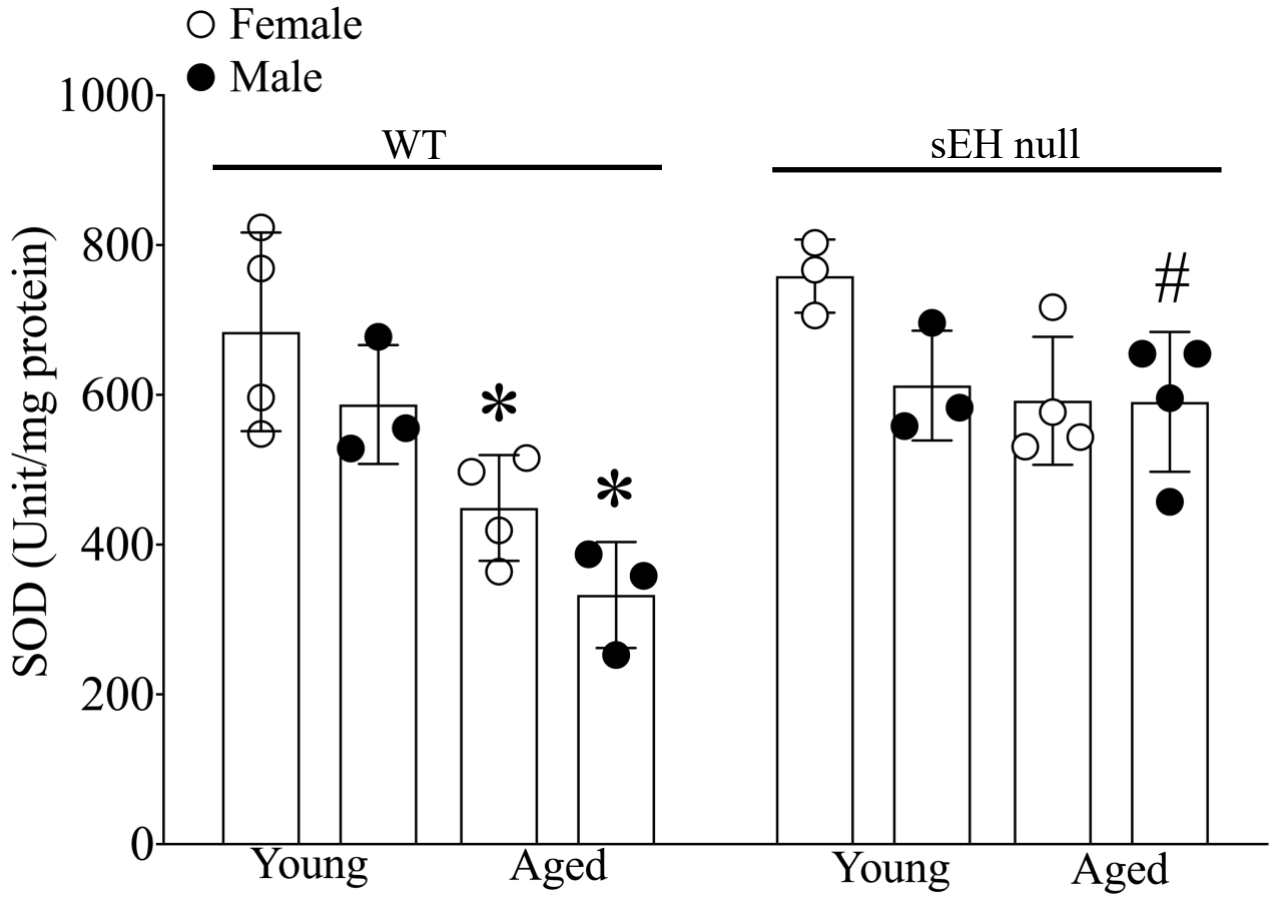

Figure 2.10. Cardiac superoxide dismutase activity in WT and sEH null mice: SOD activity in young and aged WT and sEH null mice was determined in cytosolic fractions. Data represented as mean ± SEM, n=3-4, *P* <0.05, *vs young counterparts; #vs WT counterparts.

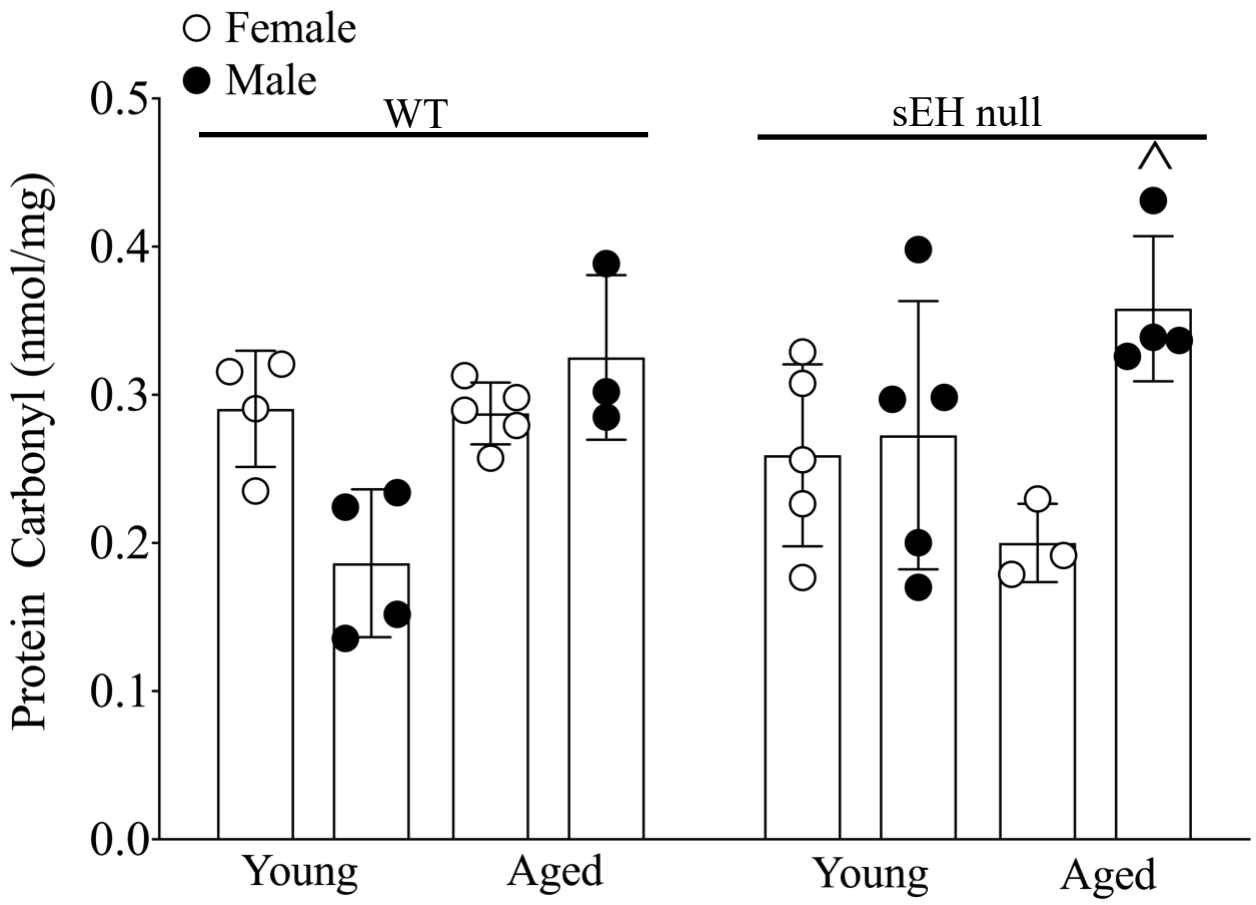

Figure 2.11. Cardiac protein carbonylation level in WT and sEH null mice: Protein carbonylation level was assessed in cardiac cytosolic fractions from young and aged WT and sEH null hearts. Data represented as mean ± SEM, n=3-5, P <0.05, ^vs female counterparts.

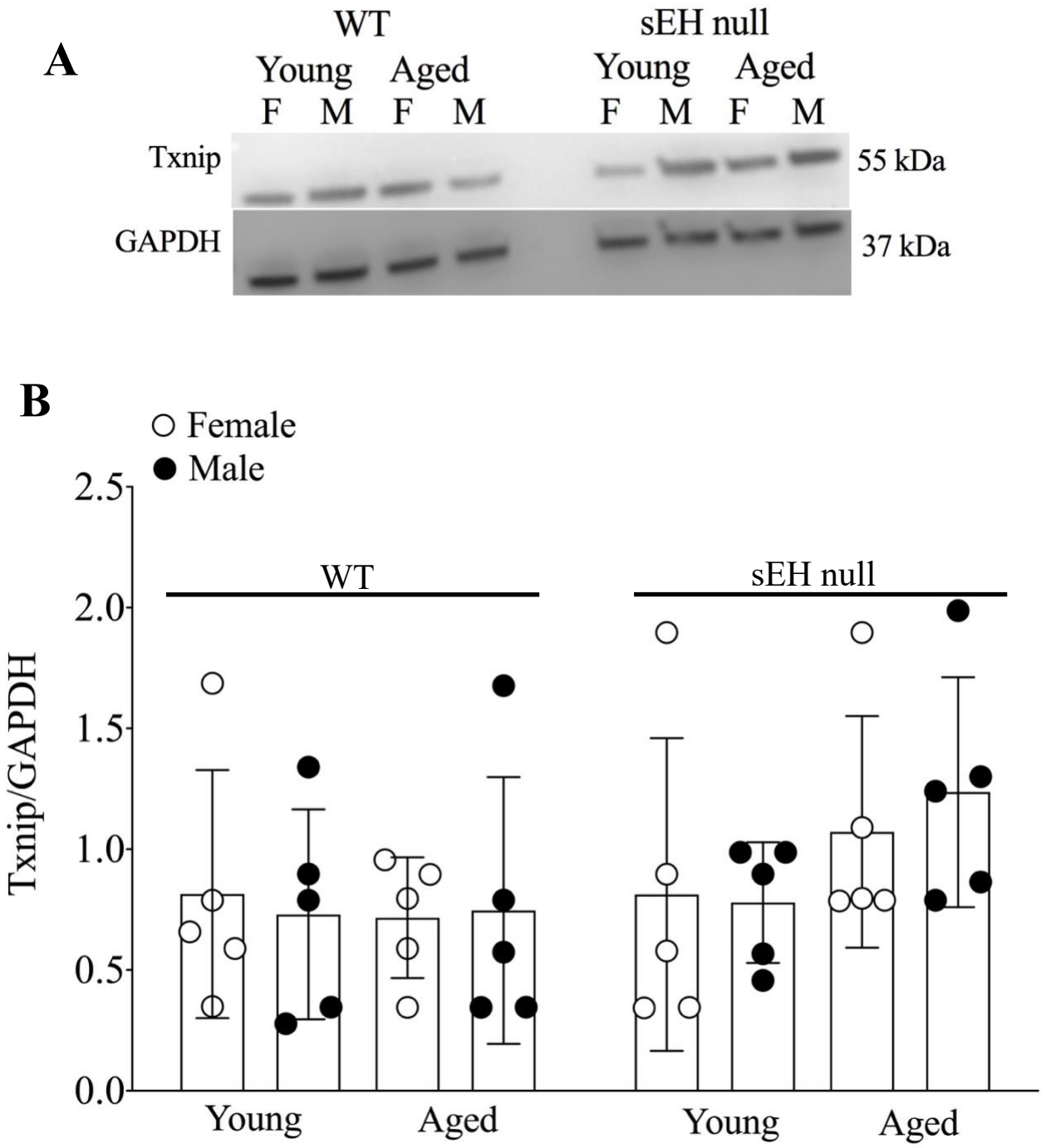

Figure 2.12. Protein expression of cardiac Txnip in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac Txnip in cytosolic fraction of murine hearts. Protein expression of Txnip was normalized to GAPDH. Data represented as mean ± SEM, n=3-5, P <0.05.

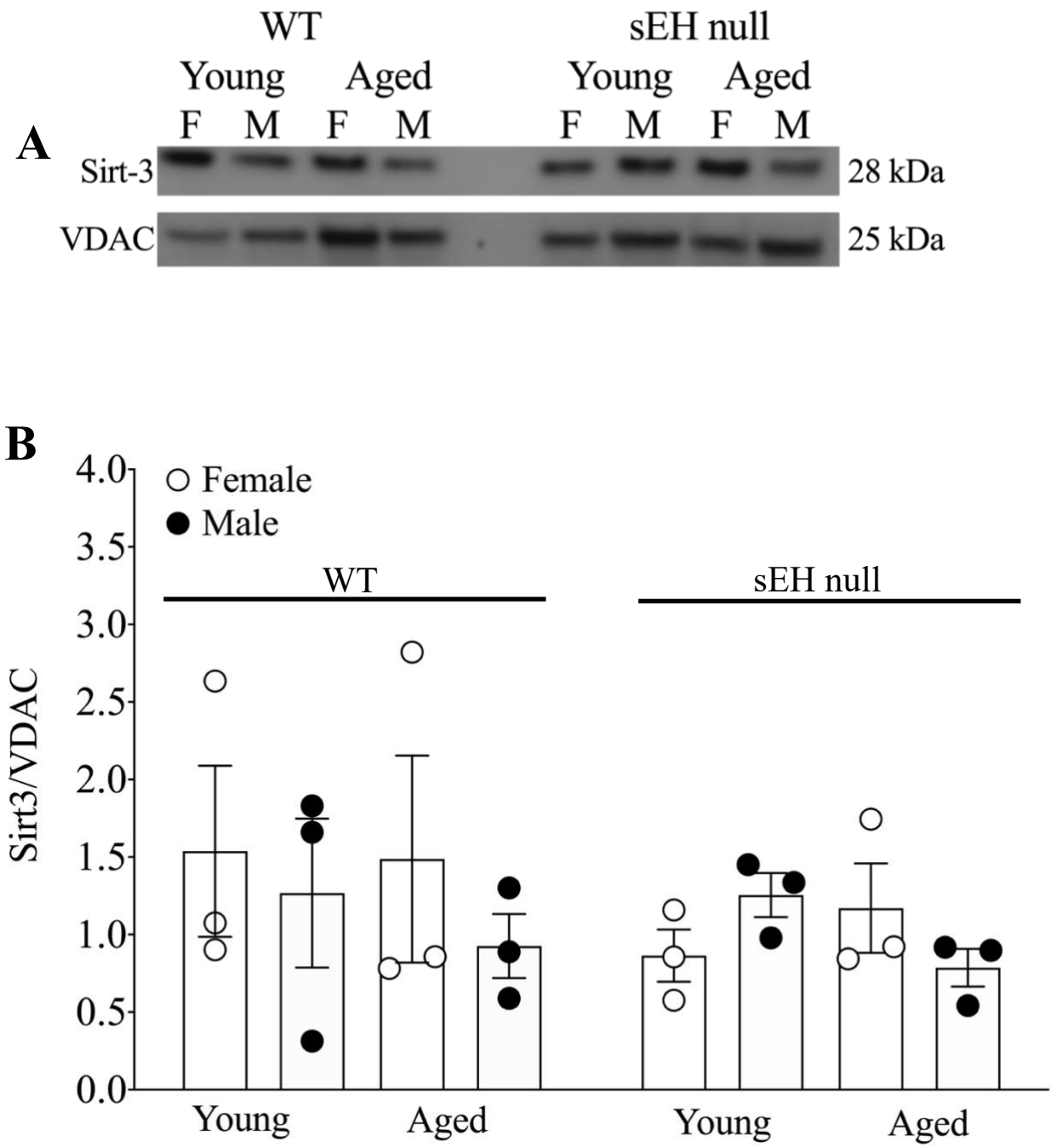

Figure 2.13. Protein expression of renal sirtuin-3 in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac sirt-3. Protein expression of Sirt-3 was normalized to VDAC. Data represented as mean ± SEM, n=3, P <0.05.

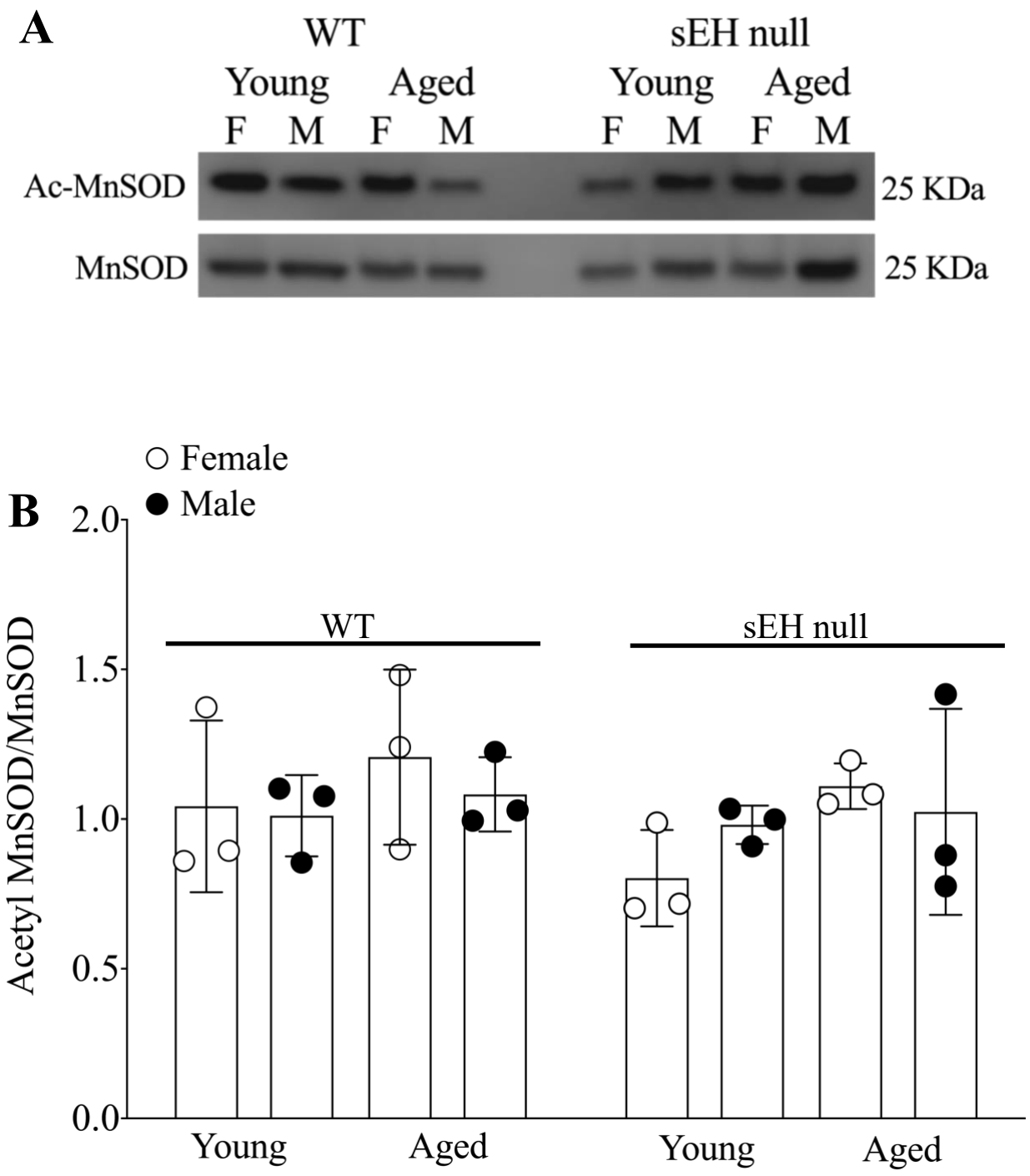

Figure 2.14. Protein expression of renal acetyl-MnSOD in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac AcMnSOD. Relative protein expression of AcMnSOD was normalized to total MnSOD in young and aged WT hearts and sEH null kidneys. Data represented as mean ± SEM, n=3, P <0.05.

2.3.4 Cardiac mitochondrial ultrastructure is preserved in aged female sEH null mice

Dysregulation of mitochondrial dynamics has been shown as a hallmark of aging leading to formation of swollen and fragmented mitochondria [218]. In the current study, there were no differences observed in Drp-1 and Mfn-2 protein expression in any group (Fig.2.15A, B; Fig.2.16A, B). To obtain an estimate of cardiac mitochondrial content, we measured the activity of citrate synthase, a rate-limiting enzyme involved in mitochondrial oxidative metabolism [219]. There were no differences in citrate synthase activity in any group suggesting the overall mitochondrial content was not significantly altered (Fig.2.17). Conventional TEM was employed to assess mitochondrial ultrastructure in the left ventricular free wall of both young and aged mice (Fig.2.18). Marked alterations in mitochondrial ultrastructure, exemplified by decreased cristae density, disturbed arrangement in the myofibrillar spaces and enlarged size, were observed in both male and female aged WT hearts (Fig.2.18E, F). The age-related changes to mitochondrial morphology were absent in sEH null animals (Fig.2.18G, H).

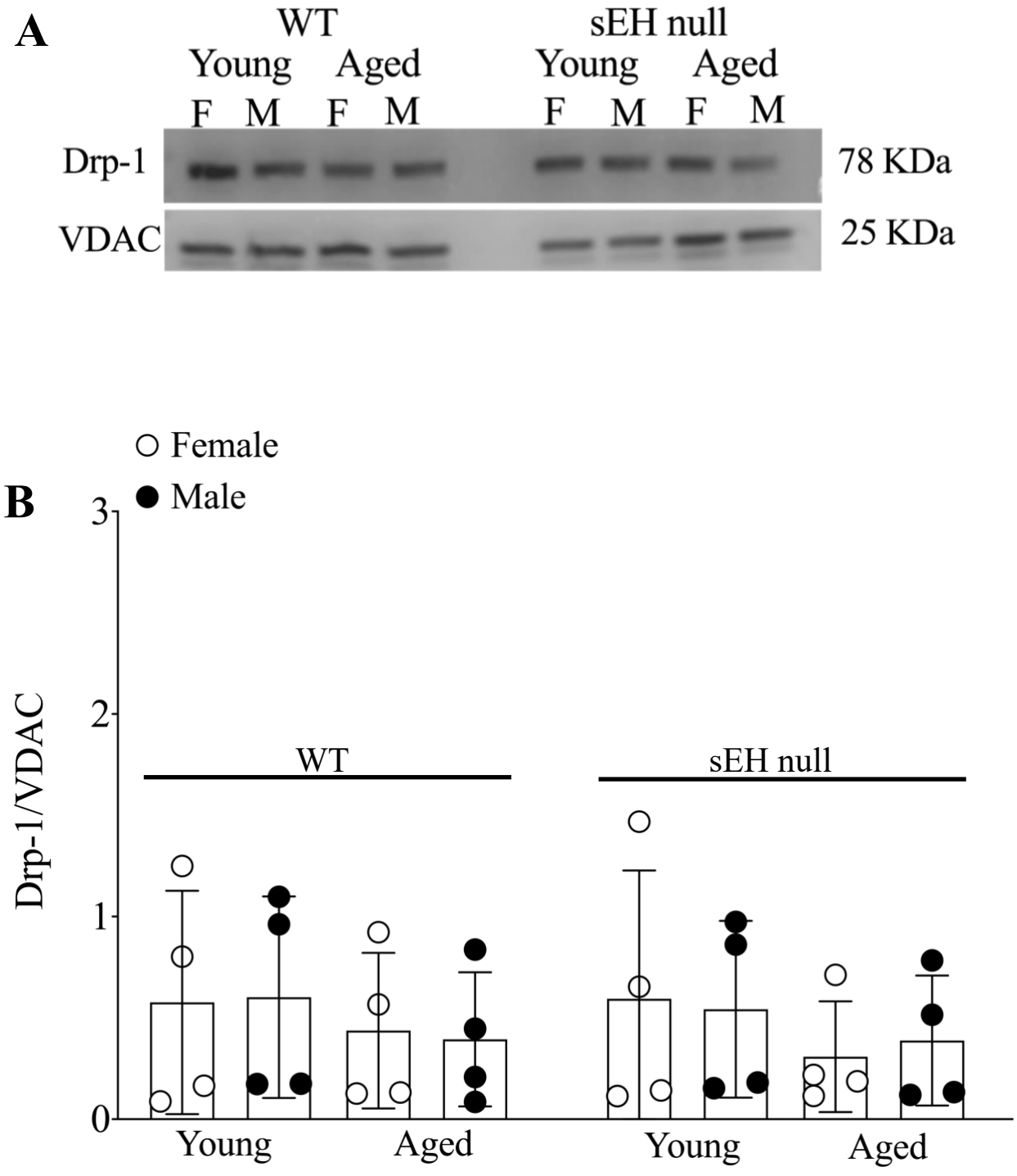

Figure 2.15. Protein expression of cardiac Drp-1 in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac Drp-1. Relative protein expression of Drp-1 was normalized to VDAC in young and aged WT hearts and sEH null hearts. Data represented as mean ± SEM, n=4, P <0.05.

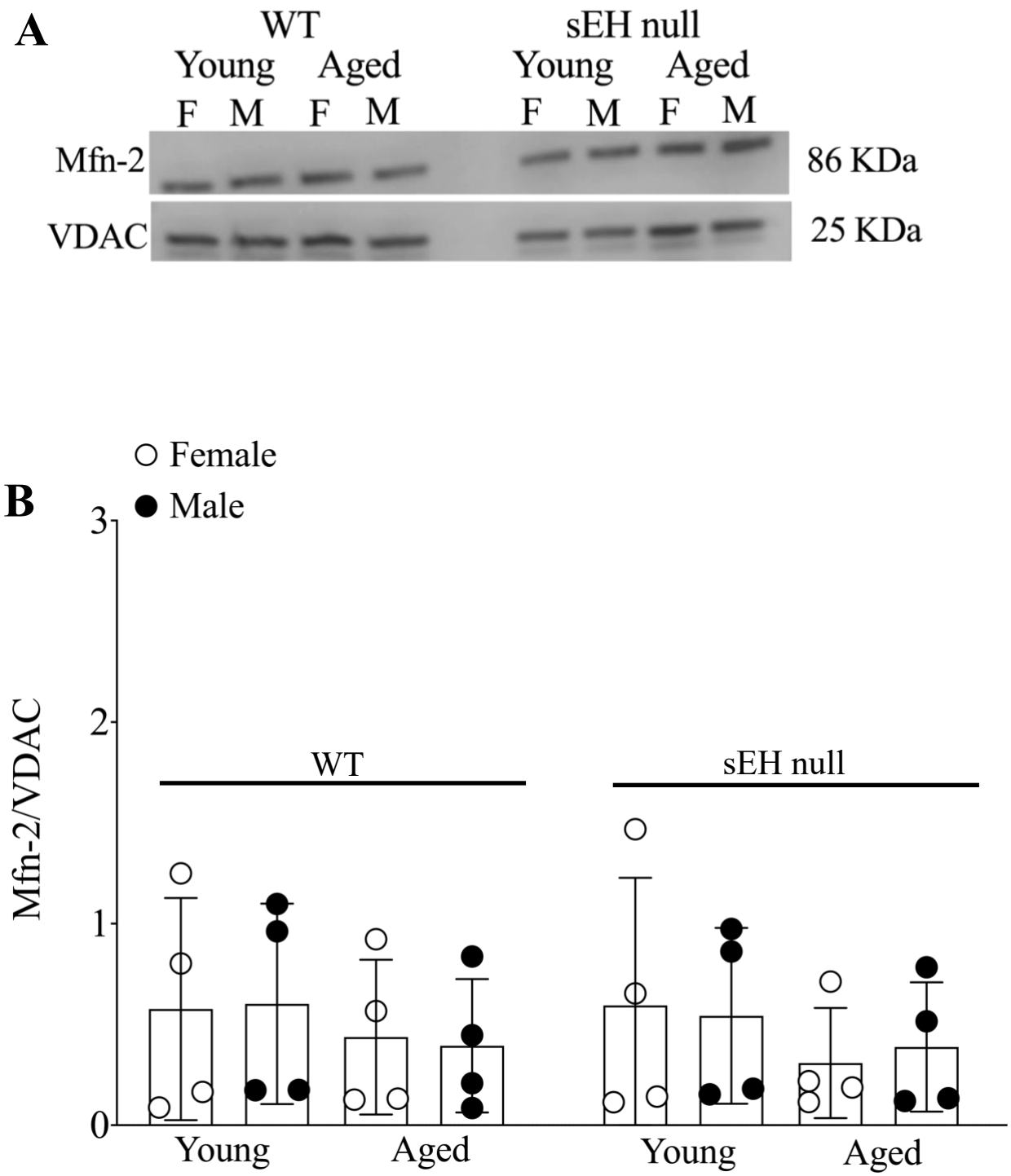

Figure 2.16. Protein expression of cardiac Mfn-2 in WT and sEH null mice: (A) Representative immunoblots and (B) quantitation for cardiac Mfn-2. Relative protein expression of Mfn-2 was normalized to VDAC in young and aged WT hearts and sEH null hearts. Data represented as mean ± SEM, n=4, P <0.05.

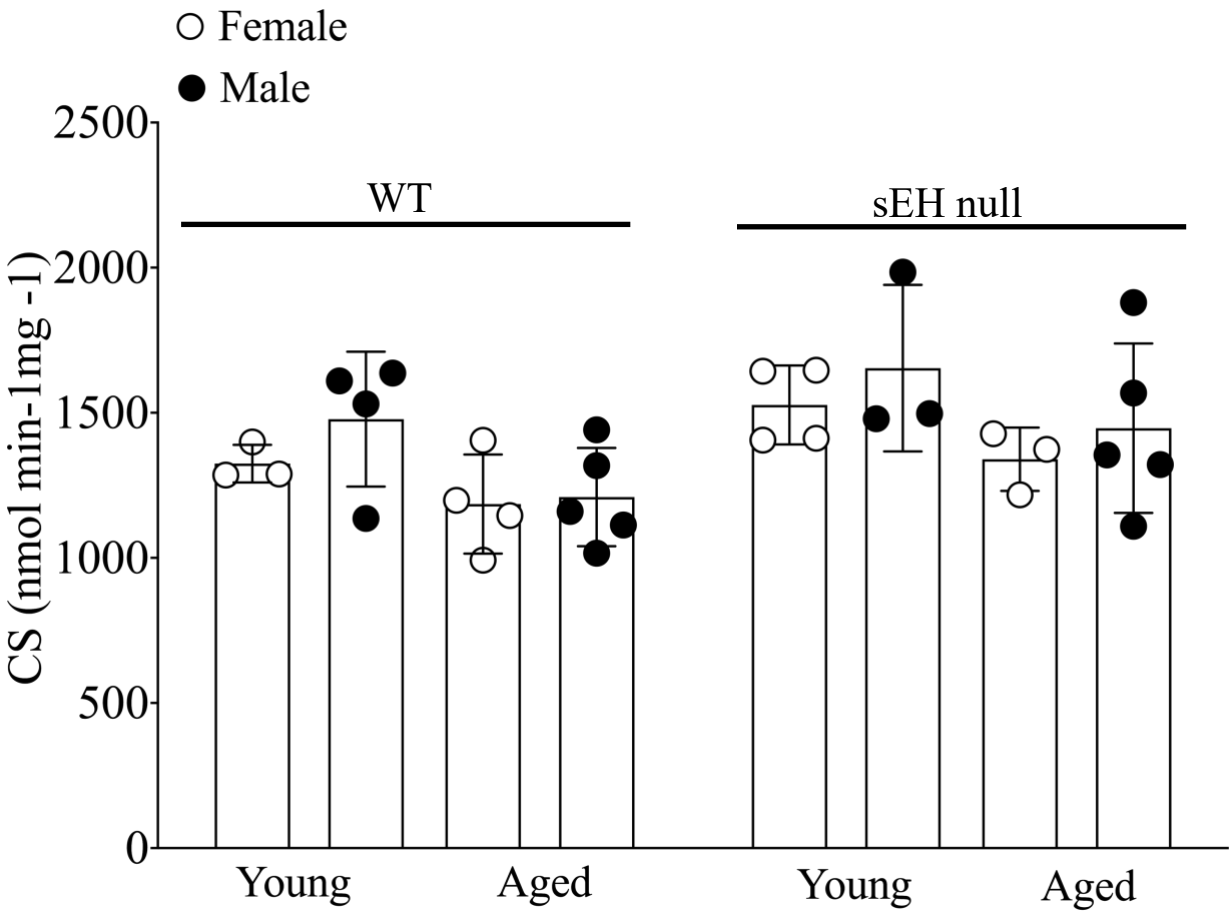

Figure 2.17. Citrate synthase activity in hearts from WT and sEH null mice: Citrate synthase activity level as a biomarker of mitochondrial content in young and aged WT and sEH null mice was determined spectrophotometrically; data are represented as mean ± SEM, n=5-8, P <0.05.

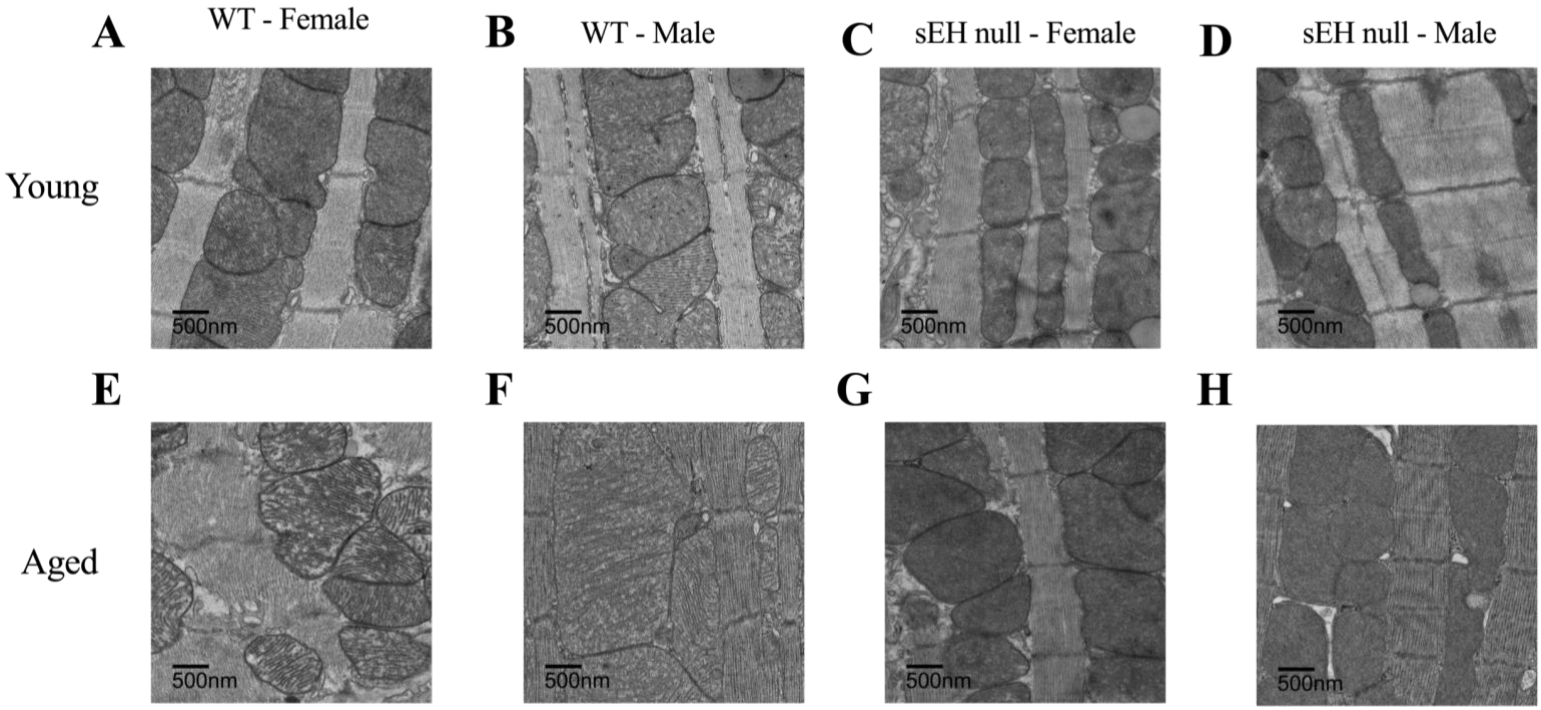

Figure 2.18. Mitochondrial ultrastructure in hearts from WT and sEH null mice: Representative transmission electron micrograph images from young (A-D) and aged (E-H) murine hearts, n=1 per group.

2.4 Discussion

Biological aging is a natural process resulting in marked changes to an individual's ability to overcome stress, which worsen adverse outcomes such as increased CVD risk [220]. Although the underlying mechanisms behind cardiac aging remain elusive, mitochondrial dysfunction is hypothesized to be a major contributor [221]. In particular, age-associated mitochondrial damage leading to increased ROS production damaging mitochondrial DNA and proteins as well affecting quality control processes, ultimately contributing to decreased cardiac function [3, 181, 222]. Importantly, sex and gender differences are known to affect the etiology, presentation and prognosis of CVDs as individuals age [157, 223, 224]. Unique CVD risks for women include the cessation of menarche (menopause), preeclampsia, gestational diabetes and certain autoimmune inflammatory disorders, such as systemic lupus erythematosus [160]. While men on average present with CVD at a younger age, evidence suggests CVD risk factors such as hypertension and diabetes play a greater role in disease acceleration in women [225]. A further understanding and characterizing potential sex-specific mechanisms contributing to cardiac aging may provide new insights for the optimal prevention and management of age-related CVDs. Consistent with literature, we report sex-differences in the age-associated development of myocardial hypertrophy and deterioration of cardiac function; however, we demonstrate novel data highlighting the beneficial effect of deleting sEH.

In humans, sex-dependent differences in cardiac aging indicate males on average exhibit greater impaired systolic function coupled with increased wall thickness, cavity dimension and LV mass [156]. Conversely women display a greater degree of diastolic impairment coupled with increased concentric remodeling, with systolic impairment

occurring later than in their male counterparts [225, 226]. Murine models are unable to replicate changes in blood pressure and blood cholesterol often present in human patients with CVD; however, they are a useful model recapitulating many human age-related changes in cardiac structure and function, such as increased LV mass, decreased diastolic filling ratios and reduced fractional shortening [38]. In the present study, we observed a significant decline in systolic and diastolic parameters coupled with a significant increase in LV mass in aged male and female WT mice. These data are consistent with what is observed clinically in aging humans. Interestingly, aged female sEH null mice demonstrated preserved systolic function and LV mass but exhibited diastolic dysfunction. Conversely, aged male sEH null mice demonstrated a significant reduction in systolic function but no significant change in diastolic parameters (Data not presented in this thesis; the cardiac function data can be found in Jamieson and Keshavarz-Bahaghighat et al, Frontiers in Physiology, 2020; [153]). Previously, we demonstrated cardioprotective effects in aged sEH null mice following myocardial infarction[227]. This previous study used combined males and females and was not designed to assess sex differences, but rather generalized aging effects in an injury model. The present data suggest important sex-specific differences in cardiac aging following sEH genetic deletion in the absence of any defined disease state.

Sexual dimorphism in sEH expression and activity has been documented in the renal, hepatic and cardiovascular systems in young rodent models [166-168, 228, 229], yet the exact mechanism(s) behind these differences remain unknown. Recent studies have demonstrated estrogen/estrogen receptor mediated methylation of the sEH promoter region causes gene silencing in female rodents [228]. This epigenetic silencing of sEH

expression may be responsible for some sexual dimorphism observed in young animal models, although whether this occurs in aged animals is unknown. In the current study, we observed an increase in sEH expression in aged WT males that was absent in WT females. In addition, there was an age-related increase in mEH expression in WT mice and sEH null females, but not sEH null males. The increased mEH expression in females may be a compensatory response to the sEH deletion but it is unknown why this does not occur in aged sEH null males. The role of mEH in cardiac eicosanoid metabolism has only recently been comprehensively investigated in vivo. Early data suggested sEH demonstrates higher catalytic ability compared to mEH and plays the predominate role in epoxylipid metabolism[196, 198, 230]. Conversely, traditionally mEH has been considered an important mediator of xenobiotic metabolism, with limited contribution to cardiac epoxylipid metabolism[198]. More recent data from Edin et al. suggests that under basal conditions it is not catalytic activity, but rather substrate availability that drives epoxylipid metabolism[231]. Cell injury such as ischemia promotes tissue damage and the release of free arachidonic acid; under these conditions sEH plays the dominate role in epoxylipid metabolism. Conversely, under basal or physiological conditions mEH may act as a "first-pass" hydrolase, acting to remove the small amount of endogenous epoxylipids produced [231]. Furthermore, while the tethering of mEH to the microsomes may hinder its ability to scavenge epoxylipids from the cytosol, if epoxy fatty acids remain bound in the microsomes the close proximity of mEH would be beneficial for the removal of these lipids. Interestingly, here we demonstrate mEH and sEH are both significantly increased across aging in WT males, with only increased mEH observed in both aged WT and sEH null females. Whether these differences over aging and between sex are related

to changes in epoxylipid formation, shifts in epoxylipid storage, or alterations in enzymatic catalytic activity remain topics of on-going study. Interestingly, here we demonstrate female sEH null mice were protected against aged-dependent development of hypertrophy and had preserved cardiac systolic function, while aged sEH null male mice were not protected against hypertrophy but demonstrated preserved diastolic function.

Mitochondria are powerful organelles essential for maintaining cardiac function through oxidative phosphorylation and ATP generation; however, they are also the main site of ROS production [232, 233]. In both mice and humans during cardiac aging ROS production outpaces mitochondrial scavenging capacity correlating with the decline in function [234, 235]. Evidence of sex specific differences in mitochondrial function and morphology have been observed in healthy and diseased states but the underlying molecular mechanisms remain poorly understood [165]. For example, cardiomyocytes from female rats have been found to possess lower mitochondrial content yet exhibit more efficient mitochondria compared to males [164]. Furthermore, female rats show lower levels of mitochondrial hydrogen peroxide in liver and brain [163]. In the current study, while we did not see any differences in level of Txnip in murine hearts of different age and sex groups, an age-related increase in the level of protein carbonylation was observed in males indicating a significant increase in cardiac oxidative stress. MnSOD is the primary mitochondrial antioxidant enzyme that contributes to maintaining mitochondrial function; moreover, inactivation of the MnSOD gene in mice results neonatal lethality [236, 237]. In rat brain and liver, higher expression and activity of MnSOD in females is associated with lower oxidative damage [163]. The activation of MnSOD is primarily

regulated through its deacetylation via Sirt-3, which is the predominant mitochondrial deacetylase [152]. Sirt-3 deficient mice demonstrate mitochondrial dysfunction and excessive production of ROS as well cardiac fibrosis and hypertrophy [75, 238]. It has been reported that the hyperacetylation and deactivation of mitochondrial proteins including MnSOD over aging is associated with a decline in Sirt-3 activity [152]. The decline in Sirt-3 activity coupled with a significant increase in expression of AcMnSOD in aged WT animals is consistent with the literature. Importantly, our data demonstrated sEH deletion preserved Sirt-3 activity in aged mice and was associated with reduced expression of AcMnSOD. Moreover, SOD activity was reduced significantly in WT mice but not in sEH null mice. Importantly, the increased antioxidant activity mitochondrial SOD observed in sEH null mice correlated with better mitochondrial ultrastructure. Recent evidence suggests Sirt-3 potentially has a role in limiting cardiac hypertrophy as it is found to be downregulated in mouse hypertrophic hearts [64, 239]. Sirt-3 mediated activation of MnSOD and subsequent ROS scavenging is proposed to suppress hypertrophic signaling, such as the PI3K/Akt pathway [240]. Interestingly, the increase in cardiac pAkt expression observed in all aged mice did not correlate with the oxidative stress or hypertrophic responses observed in the aged mice. Thus, these data suggest the genetic deletion of sEH provided a better capacity for cardiac mitochondria to limit potential aged-related damage, which was independent of an Akt pathway.

Many of the protective effects attributed to sEH gene deletion have been associated with increased epoxylipid levels, such as increased levels of epoxysatrienoic acids (EETs). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is known to mediate mitochondrial function and oxidative stress and also mediates Sirt-3

expression [241]. In models of obesity, EETs have been shown to activate PGC-1α, resulting in preserved mitochondrial structural and functional proteins associated with preserved Sirt-3 expression [242]. In the present study, while we observed no change in Sirt-3 expression, we observed a preservation of Sirt-3 activity. These data indicate there may be post-translational modification(s) of Sirt-3 associated with sEH genetic deletion and subsequent altered epoxylipid metabolism that is heretofore unrecognized and unrelated to the protective effects mediated through PGC-1α. Pillai et al. report that biophenolic compound hokoniol is capable of passing through the mitochondrial membranes to directly bind Sirt-3, improving affinity of Sirt-3 binding and utilization of NAD+, its limiting substrate, and thus preserving its deacetylase activity [242, 243]. While we do not observe any difference in Sirt-3 activity in our young sEH null animals, in the context of aging and resultant amplified oxidative stress, increased epoxylipid metabolites may preserve the deacetylase activity of Sirt-3 through direct binding, although no binding site has yet been identified. Interestingly, we observed a sex difference in Sirt-3 activity over aging in sEH null mice that has not previously been reported. While others found that Sirt-3 has a profound role in regulating mitochondrial quality control and dynamics in the context of age-related diseases [188], we did not find any differences in the protein expression of Drp-1 and Mfn-2 among our different experimental groups, regardless of their age and sex. Therefore, the exact mechanisms responsible for these age- and sex-dependent results remain the subject of a future study.

In the current study, we characterized the effect and sexual dimorphisms of sEH deletion in cardiac aging. The data demonstrated aged sEH null mice have preserved Sirt-3 activity, increased AcMnSOD levels and better mitochondrial ultrastructure compared

to WT mice. Interestingly, sEH null females had preserved systolic function [153] and no cardiac hypertrophy, while sEH null male mice had preserved diastolic function [153]. Increased expression of sEH was observed in WT males and marked increases in mEH expression where found in both genotypes. While further studies are necessary to elucidate the mechanism(s) behind these effects, the data highlight novel sexual dimorphic patterns of cardiac aging.

Chapter 3: Concluding remarks and future directions

3.1 Concluding remarks

Cardiovascular disease (CVD) remains the leading cause of mortality in both men and women worldwide [169-171]. CVDs, despite of being a principal contributor to death in all ages, predominantly occur in aged population [244]. In spite of well-established sex differences in presentation, prognosis, and myocardial responses, considerably little effort has been done to consider sex as an independent factor in prognosis, diagnosis and treatment of CVDs and most current therapeutic strategies are extrapolated from studies performed on middle-aged male animals. In this regard, sex-specific scientific research using aged animal models seems to be imperative to improve cardiovascular outcomes in the elderly population.

In this thesis, we characterized the sex- and age-specific cardioprotective properties of genetic inhibition of sEH using young and aged, male and female WT and sEH null mice in the absence of any defined cardiac injury. We demonstrated that sEH deletion induced cardioprotection against age-related cardiac alterations in a sex-specific pattern. While there was an age-induced deterioration of cardiac function (data can be found in [153]) coupled with a significant increase in LV mass in aging mice, female sEH null mice demonstrated preserved systolic function and LV mass. Conversely, male sEH null mice showed preserved diastolic function over aging with no superiority in their systolic function compared to their WT counterparts (data found in [153]).

While sexual dimorphism in sEH expression and activity has been documented in the renal, hepatic and cardiovascular systems in young rodent models [166-168, 228, 229], the role of mEH in eicosanoid metabolism has been studied only recently and sex-specific

differences in mEH expression and activity remain topic of an-going study. Here in this thesis, we observed an increase in sEH expression in aged WT males that was absent in WT females. In addition, there was an age-related increase in mEH expression in WT mice and sEH null females, but not sEH null males. The increased mEH expression in females may be a compensatory response to the sEH deletion but it is unknown why this does not occur in aged sEH null males.

While the etiology of age-related cardiac dysfunction remains poorly understood, dysfunctional mitochondria have been proven to play a fundamental role regulating a plethora of age-associated cardiac changes [135]. Mitochondrial abnormalities are hallmarks of cardiac aging accompanied by decrease in number of myocytes, increase in the size of cardiomyocytes, and over accumulation of lipids and fibrotic areas [173, 174]. Numerous studies have suggested that mitochondria-targeted beneficial effects of epoxylipids are an important component of their cardioprotective properties [92, 122, 136-140]. The data in the current thesis demonstrate that in the context of aging and consequent amplified oxidative stress, increased level of epoxylipids resultant from sEH deletion preserved the deacetylase activity of Sirt-3 coupled with decreased level of AcMnSOD and maintained SOD activity possibly associated with a better mitochondrial ultrastructure in both aged male and female sEH null mice.

Together, we found that sEH deletion confer cardioprotective effects through preservation of mitochondrial function and structure in the aging heart. While the exact mechanisms responsible for these age- and sex-dependent results remain the subject of a future study, the data in this thesis highlight novel sexual dimorphic patterns of cardiac aging.

3.2 Future Directions

1. Experiments to elucidate the mechanisms responsible for the age- and sex-dependent differences in beneficial effects of sEH deletion

Future studies should include the different signaling pathways associated with sexual dimorphism observed in the effects of sEH deletion. Recent studies have demonstrated estrogen/estrogen receptor mediated methylation of the sEH promoter region causes gene silencing in female rodents [228]. Therefore, one possible research area is validation of a reliable method to measure the level of sex hormones in mice and observe the correlation with sEH activity. Furthermore, in order to fully understand the relationship between estrogen and sEH, using murine model of menopause and measuring the activity and expression of sEH and level of epoxylipids will provide invaluable data to elucidate the role of sex hormones, particularly estrogen in sexual dimorphic patterns of the effects of sEH deletion.

2. Experiments to investigate the key mediators in mitochondria-targeted effects of sEH deletion against cardiac aging

Data from the present thesis demonstrate that sEH deletion improves the anti-oxidant capacity of aging cardiac mitochondria through preserving Sirt-3 activity leading to decreased AcMnSOD and maintained SOD activity. Future studies should include

investigating the mechanisms through which epoxylipids preserve Sirt-3 activity in order to further validate the involvement of Sirt-3 in cardioprotective effects of sEH deletion. As well as Sirt-3 pathway, investigating other signalling pathways contributing to improved mitochondrial function and structure, including mitophagy and mitochondrial bioenergetics is needed to further understand the beneficial effects of sEH deletion in regulating mitochondrial quality control.

3. Experiments to investigate both beneficial and detrimental effects of pharmacological inhibitors of sEH in the aging heart

In the present study, we only characterized the effects of whole-body genetic deletion of sEH against age-related cardiac alteration. Since the genetic model is not translational to human studies, future research is needed to investigate the effects of pharmacological inhibitors of sEH in cardiac aging. This will assist in exploring a more clinically relevant approach to understand the beneficial effects of epoxylipids and sEH inhibitors in limiting age-induced cardiac function and structural changes. Furthermore, inhibiting sEH has been shown to be associated with carcinogenesis and increased tumor growth associated with loss of essential hydrolase activity, increased epoxide level, and overaccumulation of EETs [99, 100, 102-104]. Moreover, in a study from our lab investigating on cardioprotective effects of sEH deletion against ischemia/reperfusion injury in aged mice, we observed that some of the aged sEH null animals developed tumors in various parts of the body, including the heart and liver. Therefore, further studies seem to be imperative to understand the pros and cons of both genetic and pharmacological

inhibition of sEH, particularly over a long period of time to be more relevant to the context of cardiac aging.

4. Experiments to investigate the effects of sEH-induced formation toxic diol metabolites in the aging heart

Epoxylipids are endogenously derived from oxidative metabolism of PUFAs and have been demonstrated to elicit cardioprotective properties via their anti-inflammatory, vasodilatory, and anti-apoptotic properties, as well as protective effects on mitochondria [88, 245]. The activities of epoxylipids are regulated through their metabolism by soluble epoxide hydrolase (sEH), which can increase their removal resulting in the formation of cardiotoxic diol metabolites, 9,10- and 12,13-DiHOMEs. As such, pharmacologically inhibiting or genetic deletion of sEH to increase epoxylipids bioavailability can be regarded as a therapeutic target in cardiac diseases [172]. In the present study, we observed an age-associated increase in sEH expression in male WT animals. However, we did not analyze the metabolite profile of n-3 and n-6 PUFA derived metabolites in the aged WT and sEH null hearts to explore possible meaningful patterns. Further studies are needed to investigate the metabolite profiles of the aged hearts, including DiHOMEs and assess the possible detrimental effects of DiHOMEs in aging-induced myocardial dysfunction.

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