DOCOSAHEXAENOIC ACID-MEDIATED CYTOTOXICITY IN IMMORTALIZED CELLS

by

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ABSTRACT

Docosahexaenoic acid (22:6n3, DHA) is an n-3 polyunsaturated fatty acid (PUFA) that is known to induce context-dependent cell survival, as well as apoptosis, but the exact cellular and molecular mechanism(s) remain unknown. DHA is known to act as a natural ligand for the peroxisome proliferator-activated receptors (PPARs) family of nuclear receptor, which may in turn influence cellular death. This thesis focused on the PPAR-mediated mechanistic induction of cell death, involving the accumulation of ceramide in an immortalized cell line treated with DHA. We utilized DHA treatment, with and without a specific inhibitor of PPARδ, GSK 3787. Our results validated PPARδ as a DHA target, which subsequently leads to the production of *de novo* ceramide, and apoptotic cell death. Various pathways leading to cell death were significantly attenuated with the simultaneous treatment of cells with DHA and GSK 3787, providing evidence that activation of PPARδ is vital to this process. Furthermore, we demonstrate that epoxydocosapentaenoates (EDPs) which are CYP oxidase metabolites of DHA induce a similar signaling pathway culminating in apoptotic cell death, suggesting that they are the active mediators. Our findings thus provide evidence for a novel pathway of DHA-mediated apoptotic cell death, and suggests that EDPs, and in particular 19, 20-EDP play a vital role in this process.

PREFACE

The majority of the experimental data included in this was performed jointly by Igor Zlobine and Dr. Victor Samokhvalov. Many of the assays and experiments were performed in a collaborative manner between Igor Zlobine and Dr. Samokhvalov, including: cell viability (Trypan blue) and metabolic activity (MTT), proteasome activity, western blotting, and lipid peroxidation (TBA-reactive products). Caspase-3 activity, DNA binding, and flow cytometry (Annexin V-FITC) were performed by Dr. Samokhvalov. Finally, the majority of the experiments assessing ROS generation (H_2DCF-DA), CCK-8 measurement, and all LC/MS measurements of ceramide accumulation were performed by Igor Zlobine.

DEDICATION

I dedicate this thesis to my partner Anastasia Greenberg- without your love and compassion I would not have been able to complete my graduate studies. I also dedicate it to my ever-supportive and optimistic parents, Valeri Berenchteine and Elena Zlobina.

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxynonenal
11,12-EET	11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic
20-HETE	20-hydroxyeicosatetraenoic acid
AA	Arachidonic acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
ALA	α-linolenic acid
AKT	Protein kinase B
AMC	7-amino-4-methylcoumarin
BP	Blood pressure
CD36	FA glycoprotein transporter FA translocase
CML	Chronic myeloid leukemia
COX	Cyclooxygenase
CVD	Cardiovascular disease
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
DHEA	Docosahexaenoyl ethanolamide
DOX	Doxorubicin
DTPA	Diethylene triamine pentacetic acid
EDP	Epoxydocosapentaenoic acid
EET	Epoxyeicosatrienoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GSK 3787	4-Chloro-*N*-[2-[[5-(trifluoromethyl)-2-pyridinyl]sulfonyl]ethyl]benzamide
H_2DCF-DA	2', 7'-Dichlorofluorescin diacetate
HR	Heart rate
I/R	Ischemia-reperfusion
LAD	Left anterior descending
LBD	Ligand-binding domain
LC/MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
MDA	Malondialdehyde
MI	Myocardial infarction
mitoK(ATP)	Mitochondrial ATP-sensitive K^+ channels
MOMP	Mitochondrial outer membrane permeabilization
MS-PPOH	*N*-methylsulphonyl-6-(2-proparglyloxyphenyl)
mPTP	Mitochondrial permeability transition pore
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide
PI	Propidium iodide
PL	Phospholipid
PPRE	Peroxisome proliferator response element
PS	Phosphatidylserine
PUFAs	Long-chain polyunsaturated fatty acids
ROS	Reactive oxygen species

RXR	Retinoid-X-receptor
SCD	Sudden cardiac death
sEH	Soluble epoxide hydrolase
SPT	Serine palmitoyltransferase
TAC	Total antioxidant capacity
TAG	Triacylglycerol
t-AUCB	Trans4[43Adamantan1ylureidocyclohexyloxy]benzoic acid

CHAPTER 1

INTRODUCTION

1.1 AN OVERVIEW OF n-3 PUFAs

Long-chain polyunsaturated fatty acids (PUFAs) have more than one double-bond in their aliphatic chain and mainly belong to the n-6 and n-3 class. n-3 PUFAs specifically pertains to fatty acids (FA) that contain their first double bond at some point past the third carbon (from the methyl end), and are between 18 and 22 carbons in length [1]. n-3 PUFAs are liberated from the cell membrane by phospholipase A2 [2, 3]. Once released, these free fatty acids (FFAs) can enter cells such as cardiomyocytes by protein-mediated transporters such as the FA glycoprotein transporter FA translocase (CD36), which is highly expressed within the heart and mediates the functionality of a membrane pump which facilitates this entry [4, 5]. FFAs can also enter after being released directly from the sarcolemma [6, 7].

Docosahexaenoic acid (22:6n3, DHA) and eicosapentaenoic acid (20:5n3, EPA) are the predominant biologically active n-3 PUFAs found in fish oil, which represents the main dietary source for humans [8, 9]. However, as discussed below, metabolites of n-3 PUFAs are an emerging class of FAs with a high degree of biological activity. The biosynthesis of PUFAs is a complex process. PUFAs undergo many steps during metabolism which includes synthesis and degradation, but also partial degradation-resynthesis cycles involving microsomes and peroxisomes [10]. α-linolenic acid (18:3n3, ALA) is a plant-derived n-3 PUFA commonly found in vegetable oils such as rapeseed

and flaxseed that can be converted into both EPA and DHA [10, 11] (Figure 1.1). ALA can be converted to EPA via increases in carbon chain length by desaturation, elongation, and further desaturation, by Δ6 desaturase, elongase and Δ5 desaturase enzymes, respectively [12]. EPA can then be further processed and metabolized by elongase and Δ6 desaturase into DHA [8, 11, 12]. Although this conversion of ALA into EPA and DHA does occur within the endoplasmic reticulum, it is an organ-specific process that is not very efficient, especially the conversion of ALA into DHA [3, 11, 13-16]. Consequently, the majority of circulating DHA is a direct result of dietary intake, which underscores the fact that it is considered an essential FA vital for well-being [13, 17, 18].

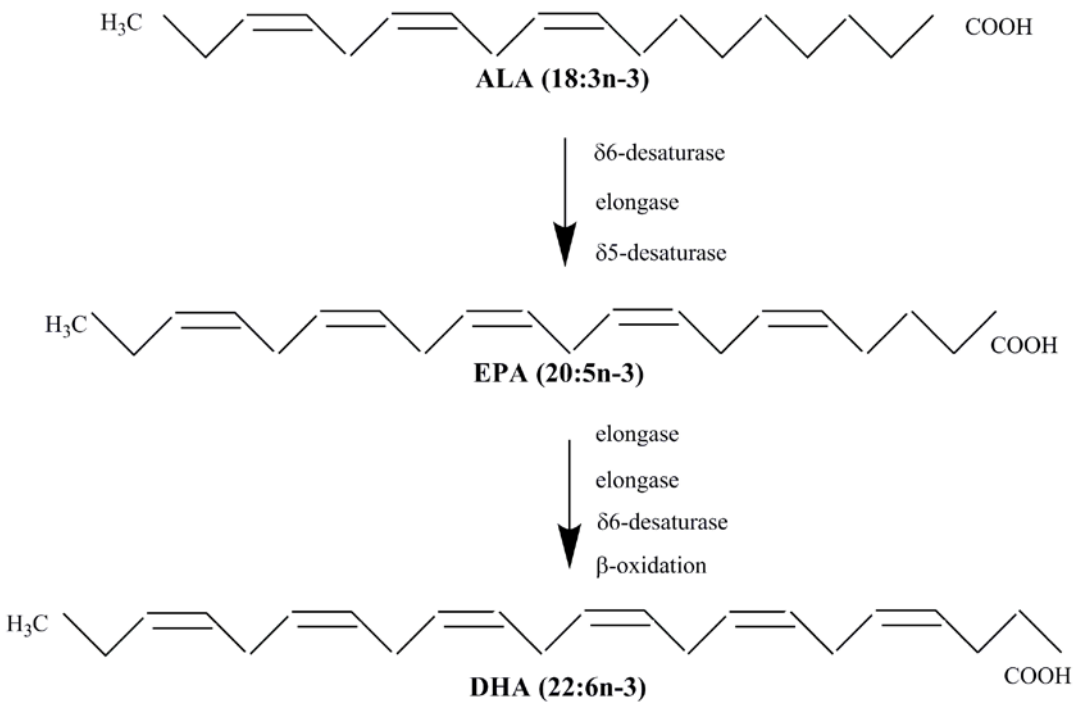

Figure 1.1 Generalized schematic of n-3 PUFA conversion (adapted from ref 30).

ALA is desaturated, elongated, and desaturated again to create EPA. EPA then undergoes two cycles of elongation, followed by saturation and β-oxidation, eventually becoming DHA.

The recommended daily intake of n-3 PUFAs differs between organizations. For example, European authorities recommend a minimum of 0.5 g/d [1], while the World Health Organization recommends 0.25 g/d of EPA and/or DHA [19]. Epidemiological data suggests that 3-4 g/d may be optimal [12]. On average, a minimum daily intake of ~0.25-0.5 g/day EPA and/or DHA is the standard recommendation [20, 21]. However, modern 'Western' diets are at the lower end of this spectrum typically providing only 0.25 g/day, of EPA and DHA mainly by way of oily fish such as mackerel, salmon, and trout [3, 12, 16, 22]. In addition, n-3 PUFAs can be obtained from alternative sources such as microalgae, which is primarily supplied by phytoplankton [23]. As previously mentioned, plant sources such as flaxseed oil, rapeseed oil, and soy oils, along with flax seeds and chia seeds can also be used as sources of n-3 PUFAs [8, 24, 25].

An early epidemiological study provided evidence for the potential benefits of n-3 PUFAs. This landmark study showed that an increased intake of n-3 PUFAs in Greenland Inuits was linked to a lowered incidence of myocardial infarction (MI) [26]. This is now an established relationship, whereby an increased intake of n-3 PUFAs is associated with a reduction in the risk factors, and the incidence of cardiovascular disease (CVD), with particularly potent prevention of sudden death and primary cardiac arrest [27-30]. While the exact mechanisms remain unknown, evidence suggests that the PUFA-mediated mitigation of CVD risk involves protective effects toward endothelial function [31], reduction in circulating triacycleglycerides [32 33] and anti-arrhythmic effects [34 35].

Furthermore, n-3 PUFAs also possess properties that help in the stabilization of membranes, and have the potential to help treat cardiac arrhythmias [36], reinforcing their vital role in the well-being of the cardiovascular system. To date, there have been more than 500 clinical trials conducted looking at the beneficial roles of n-3 PUFAs [37]. Indeed, a more recent large prospective Japanese study found that the intake of n-3 PUFAs was inversely related to CVD, and heart failure in particular [38].

1.2 n-3 PUFA METABOLISM

As opposed to the cyclooxygenase (COX) and lipoxygenase (LOX) pathways of metabolism, the cytochrome (CYP) P450-mediated metabolites of EPA and DHA are not as well characterized. However, it is now known that the CYP P450 superfamily of enzymes catalyze the oxidation of endogenous and exogenous substrates such as FAs, lipids, and drugs, and they are responsible for the generation of oxidase metabolites of DHA, referred to as epoxydocosapentaenoic acids (EDPs). In particular, CYP epoxygenation leads to the conversion of DHA into six regioisomeric metabolites (4,5-, 7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP), which are hypothesized to act as lipid mediators [39, 40]. Metabolites of DHA are an understudied area of research, but their importance is beginning to be recognized. Indeed, EDPs have been shown to play a key role in a multitude of cellular and physiological processes, with

demonstrable effects on paracrine as well as autocrine signaling [41-43]. In addition, these oxidase and epoxy metabolites have been shown to act as both anti-angiogenic and anti-inflammatory agents, as well as potentially providing protection against the development of Alzheimer's disease [42, 44, 45], pointing to the notion that they engage in system-wide signaling. Furthermore, studies showing protection against cardiac arrhythmias have attributed this to the actions of EDPs, which was previously recognized as a function of DHA-mediated signaling [46, 47].

There are many EDP methyl ester regioisomers, but 19,20-EDP is relatively resistant to degradation by soluble epoxide hydrolase (sEH) and is emerging as a particularly interesting and powerful lipid mediator in CVD [42]. In particular, DHA monoacylglyceride is metabolized into 19, 20-EDP via CYP P450 within human pulmonary arteries, and those elevations in 19, 20-EDP lead to a down-regulation in Ca^{2+} sensitivity and active tone, making it an important metabolite for patients with pulmonary hypertension [48]. There is a connection between low levels of DHA and its associated metabolites with the development of hypertension [49], and mice given 19,20-EDP display antihypertensive effects in an angiotensin-II dependent manner [47].

The potency of DHA metabolites has previously been shown using a porcine model in coronary arterioles, whereby 19, 20-EDP was ~100,000 fold more potent than DHA [43]. 19,20- EDP was also shown to play an intimate role in the prevention of tumor growth and metastasis [45]. This suggests the

interesting possibility that metabolites may be more effective in treating CVD, and potentially cancer, and could potentially be used in the future in order to help to clear up inconsistencies in clinical trials carried out with n-3 PUFAs showing positive results [50-52] , while others demonstrate neutral or even detrimental effects of fish oil supplementation in cancer patients [50, 53].

Under typical cellular culture conditions there is auto-degradation and endogenous metabolism of parent compounds such as DHA. Indeed, this was shown experimentally with the results suggesting that PUFAs with more double bonds are also more susceptible to autoxidation (i.e. DHA more so than EPA) [54]. Accordingly, in terms of lipid peroxidation this could be detrimental, but it also allows for an interesting hypothesis to be put forth that many of the previously published positive effects of n-3 PUFAs, and in particular DHA, could potentially be attributed to the actions of their more biologically active and potent metabolites such as EDPs.

n-3 PUFAs can also be metabolized into a vast array of signaling molecules and mediators, such as of resolvins, protectins, maresins, and nitrolipids/oxylipds. These metabolites possess inhibitory effects towards reactive oxygen species (ROS), as well as towards the pro-inflammatory cytokines [49]. An interesting class of n-3 PUFA derived lipid mediators are resolvins, with the E and D series of resolvins derived from EPA and DHA, respectively [55]. Using the H9c2 rat cardiac cells, it was shown that the EPA-derived resolvin E1 (RvE1) provided dose-dependent protection against

apoptosis both under normoxic and hypoxic conditions, which was partially attributed to a dampening of caspase-3 activity [56]. A protective effect was also shown in this study in rats that had undergone ischemia-reperfusion (I/R), in which RvE1 dose dependently reduced infarct size [56]. Another class of metabolites as shown by Yang et al. (2011) are the docosahexaenoyl ethanolamide (DHEAs), which are bioactive oxygenated forms of DHA that were shown to have a protective effect toward organs and display anti-inflammatory properties. This suggests that these metabolites may potentially be effective in preventing I/R-induced injury [57]. Overall, this data points to a vital role for DHA metabolites in both the cardiovascular system, as well as in protection against cancer.

1.3 DIFFERENTIAL EFFECTS OF DHA AND EPA IN CARDIOVASCULAR DISEASE AND CANCER

Significant differences in study duration, dosages and endpoints measurements have contributed to the discrepancy in data interpretation in studies looking at the influence of n-3 PUFAs towards CVD. Additionally, DHA and EPA are known to display differential effects towards various diseases. For example, a meta-analysis of clinical trials demonstrated that EPA was the key PUFA in fish oil supplement given to patients for the treatment of depression [58]. Overall, EPA was more efficacious as compared to DHA in preventing platelet aggregation in men [59], and EPA fed to rats for 6 weeks significantly increased post ischemic recovery of aortic flow, with a concomitant accumulation of EPA within membrane phospholipids [60]. EPA is incorporated into erythrocyte membranes within 48 h, while DHA can take up to ~4 weeks [61], suggesting important time-dependent differences between these two n-3 PUFAs.

DHA is also known to mediate specific cell signaling effects, as DHA (4g/d) for 6 weeks was particularly efficacious in lowering blood pressure (BP) and heart rate (HR) in a group of overweight hyperlipidemic men [62]. A recent study looked at the direct influence of DHA on the heart, instead of its well-established role in reducing CVD risk factors. Rat hearts were protected from I/R when DHA was infused directly into the heart before and after injury, which

occurred via the Notch signaling pathway [63]. Furthermore, DHA treatment is particular effective in reducing the propensity for Ca^{2+}-induced mitochondrial permeability transition pore (mPTP) opening (mPTP importance discussed below) in cardiac mitochondria in rats when given at 2.5% of total caloric intake for 8 weeks. Although not directly assessed, it appears that the n-6/n-3 ratio (discussed below) was lowered as well, as DHA feeding lead to increased phospholipid incorporation of both DHA and EPA, with a simultaneous significant decrease in arachidonic acid (AA) [64]. Some evidence suggests that DHA is more efficacious than EPA in reducing the progression of tumors in colorectal cancer [65]. DHA has also been shown to induce apoptotic cell death in triple negative breast cancer as detected by Annexin V-FITC/PI binding, with a concomitant upregulation of caspases and ROS [66]. These results point to EPA and DHA-specific signaling which can be time, and cell-dependent, and require further research to clarify their potential divergent roles.

1.3.1 CLINICAL TRIALS UTILIZING n-3 PUFAs

Inconsistencies and inconclusive data exist when looking at clinical data, as most, but not all studies looking at potential beneficial effects of n-3 PUFA supplementation agree. A meta-analysis of randomized clinical trials did not find any correlation between n-3 PUFA intake and the incidence of sudden death or MI [67]. The OMEGA trial found that n-3 PUFAs ethyl esters (1g/d)

were not effective in reducing sudden cardiac death or total mortality in German patients post MI, as compared to a control group in a randomized, double-blind trial after 1 year of treatment [68]. This is in opposition to results from the GISSI trial which demonstrated that n-3 PUFA supplementation did decrease the risk of cardiovascular death [69]. The age of patients in clinical trials is an important factor to consider. For example, the age of participants in the OMEGA trial were ~10 years older than those in the GISSI trial [70], suggesting that supplementation with n-3 PUFAs may be less affective in older populations. The source of n-3 PUFAs given to patients in clinical trials varies greatly [70]. In addition, the length of intervention varies, but a relatively fast response can be observed, as clinically relevant decreases in sudden cardiac death (SCD) have been observed after ~4 months of supplementation [69].

1.4 IMPORTANCE OF THE n-6/n-3 RATIO

There is a complex interplay and competition amongst n-3 and n-6 PUFAs. n-3 PUFAs can compete with n-6 PUFAs such as AA for enzymatic metabolism and subsequent decreases in n-6 PUFAs are associated with anti-angiogenic and anti-inflammatory effects [71, 72]. This means that n-3 and n-6 PUFA levels influence one another, and an excessive amount of n-6 PUFAs can preclude n-3 PUFA metabolism, and vice versa [73].

The n-6/n-3 FA ratio which has been utilized as an indicator of health status (or disease), as opposed to reporting their individual values alone due to factors such as competition for mutual metabolizing enzymes and protein targets [74, 75]. From an evolutionary perspective, humans are thought to have typically consumed equal amounts of n-3 and n-6 PUFAs [76]. Furthermore, it is suggested that the risk for development of CVD as a result of changes to the n-6/n-3 PUFA ratio is a relatively recent trend in terms of historical food consumption [75, 77]. A high n-6/n-3 FA ratio has been associated with a wide range of pathologies, including acute coronary syndrome, prostate cancer, hypercholesterolemia, inflammation, and coagulation [74, 78-82]. Current evidence suggests that a ratio of 1:1 may be optimal to mitigate CVD issues [75, 83]. Other work using a rat model of colon carcinogenesis, showed that as compared to a 1 to 1 ratio, a n-3/n-6 ratio of 2.5:1 (n-3 PUFAs compared to corn oil) led to significantly improved chemoprotective benefits [84]. One strategy to increase DHA and EPA levels in order to facilitate the anti-cancer and cardioprotective benefits that they bring is by lowering the n-6/n-3 ratio [73]. Thus, studies solidifying the recommendation for the optimal n-6/n-3 FA ratio are continuing to support the use of this ratio as a prognostic marker for CVD development and treatment, and with additional studies, its validity may also be extended to cancer treatment in the near future.

Dietary DHA is particularly effective in decreasing the amount of AA [60], and significant changes to the n-6/n-3 FA ratio occur in mice after being fed a

diet rich in either EPA or DHA for 6 weeks [60]. A proof of concept paper found that the levels of 19, 20-EDP are elevated in human plasma samples after they are given a diet high in n-3 PUFAs (4g/day) for a period of 4 weeks [85]. In addition, this increase in n-3 oxylipins coincided with a concomitant decrease in the levels of n-6 oxylipins [85].

1.5 n-3 PUFAs IN CELL DEATH

1.5.1 APOPTOTIC CELL DEATH INDUCED BY n-3 PUFAs

Apoptosis is typically described as a form of programmed cell death in multi-cellular organisms that necessitates active cellular commitment, and cells require the continuous presence of survival signals in order to avoid committing to it [86-90]. A central component of cellular fate is the metabolic activity and flux of mitochondria, as they are involved in central processes such as oxidative phosphorylation, as well as their well-known role in intracellular ROS production [86]. Mitochondria are in a constant state of dynamic flux between fission and fusion, and bioenergetics, which ultimately influences cell death processes [86]. n-3 PUFAs are known to induce cell death in various cancer cell types. A crucial step in cell death processes such as apoptosis and necrosis is the opening of mPTP, which is a channel composed of multiple proteins spanning the inner and outer mitochondrial membranes [91] that has been described as a 'self-destruct system' [92]. In spite of many attempts to define it, the exact components of the mPTP remain elusive [91, 92]. Recent developments support the notion that n-3 PUFAs have potent anti-cancer properties, whereby they induce cell death and inhibit the progression of tumors [93, 94]. It was recently shown that n-3 PUFAs inhibit the growth of cancer stem cell spheres by the induction of apoptosis as measured by an increase in annexin V expression [95].

1.5.2 n-3 PUFAs AS AN ADJUVANT IN CANCER THERAPY

n-3 PUFAs can be used in cancer as synergistic drugs or chemotherapy-sensitizers, which could potentially improve clinical results [96]. DHA has the ability to sensitize medulloblastoma tumor cells to undergo apoptotic cell death after etoposide treatment by down-regulating PI3K/MAPK signaling [97]. DHA was shown to produce a potent doxorubicin (DOX)-sensitizing effect in breast cancer cells, by upregulating total lipid peroxidation [98, 99]. A fish oil emulsion (equivalent to 20.5 μM DHA and 24 μM EPA) induced apoptosis in colorectal adenocarcinomas cells in conjunction with 5-fluorouracil via Bax signaling with a significant elevation in mitochondrial membrane depolarisation [100], which is well-known to induce apoptotic cell death [101-104]. DHA has been shown to dose-dependently enhance cytotoxic death via Her-2/*neu* in human breast cancer cells [105]. DHA treatment worked synergistically, leading to increases in potency as high as 13-fold when combined with Taxol [105]. DHA combined with epirubicin anthracycline chemotherapy had complementary roles in the induction of lipid hydroperoxides [106], which are known to induce apoptotic cell death in cancer cells [107]. DHA accumulates in a dose-dependent manner within the phospholipid (PL) and triacylglycerol (TAG) fractions within tumors of rats fed diets rich in DHA [108]. Moreover, plasma PL levels were shown to be significantly correlated with DHA levels within tumor PL, and thus plasma can be used as a more easily attainable surrogate measure for tumor levels of DHA [108]. n-3 PUFAs can also sensitize advanced stage human breast carcinomas to

various chemotherapeutic drugs. Importantly, PUFA levels were highest in patients with the most tumor regression, with DHA shown to be the most robust predictor of prognosis [109].

There is an important interaction which often occurs between cancer and CVD, whereby it is well known that the use of some chemotherapeutic drugs such as DOX can cause dose-dependent toxicities within the heart and within the cardiovascular system such as hypertension, congestive heart failure, ischemia, and arrhythmias, ultimately leading to morbidity and mortality [110-113]. Furthermore, chemotherapeutic drugs lead to long-term complications, as adults who survived a childhood cancer end up having a 15-fold increased risk for congestive heart failure [114], leading to the suggestion that there is no safe dose of DOX for children [112]. There are many other potentially cardiotoxic chemotherapeutic drugs other than DOX, which include those that fall into the categories of anthracyclines (which DOX is a part of), taxanes, alkylating agents, and HER2 receptor inhibitors, although less toxicities are typically observed with targeted therapies [112, 113, 115]. However, a recent study demonstrated that H9c2 cells which were pre-treated with 50 μM of DHA for 24 h led to the prevention of cardiotoxic increases in ROS generation [111]. It is evident that the role that DHA plays in the interconnection between cardiotoxicity and cancer is complex, and future studies will need to deduce DHA-mediated signaling for both pathophysiologies.

1.6 PPARs: AN OVERVIEW

1.6.1 PPARs METABOLISM & ACTIVATION

The first peroxisome proliferator-activated receptor (PPAR) was cloned in 1990, and PPARs are comprised of three isoforms; α (NR1C1), β/δ (NR1C2), and γ (NR1C3) [116, 117]. PPARs are a class of structurally conserved, ligand-activated nuclear receptor transcription factors, which are members of the soluble nuclear hormone receptor superfamily known to display cell-specific actions [118] [116, 119-124]. They are known to play a role in upregulating the amount of fatty acid catabolism, as well as influencing glucose and lipid homeostasis, and beta oxidation [125, 126]. Although they have differing physiological effects, all three of the PPAR isoforms possess comparable functional characteristics, and all play a vital role in glucose and lipid metabolism, as well as insulin resistance, and suppression of inflammation [118, 119, 127, 128]. In addition, some cross-talk exists between PPARs and other transcription factors via trans-repression [129-132], as well as amongst all three PPAR receptor isoforms, as it has been shown *in vitro* that PPARα and PPARγ gene expression can be transcriptionally repressed directly by PPARδ signaling, suggesting that it can function as a 'gateway' receptor [133].

A conformational change in the protein structure of PPARs is induced upon ligand binding, leading to the removal of co-repressors [121, 128]. However, in order to induce transcriptional activation for any of the PPAR isoforms, they

must associate and form heterodimers with the retinoid-X-receptor (RXR). This is followed by binding at the sequence motifs TGACCT and TGTCCT (in the promoter region), termed the peroxisome proliferator response element (PPRE), which allows for activation and downstream transcriptional regulation [118, 128 121 134]. Conversely, when ligands are not present to initiate activation, the PPAR-RXR heterodimers form complex with various co-repressor proteins, sequestering the sequence motifs in the promoter region from PPAR-RXR, ultimately blocking transcriptional activation [128, 135-138]. In addition, PPAR receptors contain a ligand-binding domain (LBD), which is larger in comparison to other nuclear receptors. This could partly explain isoform specificity, as well the ability of PPAR receptors to bind many types of lipophilic molecules [116, 132, 139]. PPARs are particularly sensitive to ligands such as unsaturated FAs [140]. PPARs can also be stimulated by synthetic compounds [123, 139], providing the basis for the use of PPARδ antagonists such as 4-Chloro-*N*-[2-[[5-(trifluoromethyl)-2-pyridinyl]sulfonyl]ethyl]benzamide (GSK 3787) [141]. GSK 3787 acts by covalently modifying cysteine 249 in the ligand binding pocket, leading to irreversible antagonism [141]. Furthermore, GSK 3787 is chemically stable, has a strong pharmacokinetic profile, it effectively blocks downstream gene expression, and is suitable for use both *in vitro*, and *in vivo* [141].

It is important to recognize that when bound by a ligand, PPARδ protein levels are maintained at a high level because they are prevented from being

degraded via proteasomes, which is important in cases of highly upregulated PPARs via ligands [116]. PPARδ is encoded by genes located on chromosome 6 in both mice and humans [116]. PPARδ has been the most understudied in terms of its mechanisms of post-translational modification and phosphorylation [118]. Of note, PPARδ mRNA is the most widely and ubiquitously expressed isoform (originally thought to be a housekeeping gene) [142], with levels typically higher than the other two isoforms [118, 143, 144]. Although widely expressed, higher levels of PPARδ are found in the intestines, liver, adrenals, keratinocytes and notably, within the heart [142 145 146 147].

Approximately a decade ago the creation of synthetic PPARδ agonists such as GW501516 that represent very potent *in vitro* and *in vivo* ligands allowed for more in-depth experimentation of this receptor [148, 149]. This was facilitated by efficient synthesis, as well as the high degree of selectivity of the PPARδ isoform over PPARα and PPARγ, as the EC(50) value was 1000-fold more selective towards it [148, 149]. These agonists have been shown to display anti-obesity and anti-atherogenic properties, as well being beneficial for dyslipidemia and insulin resistance [150-152]. In addition, there is a reduction in PPARδ within the heart in pressure-overload hypertrophy, while its activation can prevent hypertrophy in rat neonatal cardiomyocytes [153, 154].

1.6.2 PPARs IN DISEASE

Macrophages are well known for their ability to phagocytose dead cells, allowing for the completion of programmed cell death. This is important, because if dead cells are not properly removed it can lead to secondary necrosis and inflammation, and ultimately contribute to cellular death in surrounding areas [90, 155, 156]. PPARδ can act as a transcriptional sensor, identifying and removing damaged cells via macrophages, thus preventing potential autoimmune attacks [156]. A similar role was also shown for the oxysterol-activated nuclear receptor, the liver X receptor (LXR) [157], which is important because PPARδ and LXR have been shown to interact and function with one another in the regulation of cholesterol transport, energy homeostasis, atherosclerosis, systemic lupus erythematosus, inflammation, and fatty acid oxidation [144, 158-161]. Ultimately, after the activation of the LXR and PPARδ transcription factors, there is an increase in soluble ligands and cell surface receptors on macrophages, thus providing additional cellular signalling leading to an accelerated removal of apoptotic cells [144]. Importantly, after being activated by ligands, PPARs are known to respond in a cell-specific manner [118]. This suggests that their role in previously elucidated pathways cannot necessarily be extended to all cell types and models, underlining the importance of finding the specific cell signaling pathways by which they act. In cell types such as T lymphocytes, PPARδ was shown to be overexpressed, where activation of PPARδ led to increased proliferation [162]. Although, this

prolonged the survival of T cells via the ERK signaling pathway [162], this study suggests that in some cases it may be possible for excessive stimulation of PPARδ to induce proliferation, and uncontrolled stimulation may be involved in pathogenesis.

PPARγ and PPARα are predominantly located within the nucleus when not bound by ligands [163, 164], whereas PPARδ is primarily expressed in the cytosol of epithelial cells, as well as tumors cells from lymph node metastases [165]. Mice expressing an adipose-specific viral protein fused to PPARδ displayed reductions in lipid droplet size and overall fat stores. While lacking PPARδ were highly prone to the development of obesity in response to a high-fat diet. These findings firmly link PPARδ to the prevention of obesity [166]. Similar results were found in skeletal muscle [167] and the liver [168], whereby PPARδ was linked to the prevention of obesity, hyperglycemia and insulin resistance [167, 168]. In some studies activation of PPARδ has been shown to be protective against cancer. A recent paper demonstrated that PPARδ suppresses RAS-dependent tumorigenesis [169]. The activity of PPARδ appears to be cell type-dependent, as it has recently been demonstrated that PPARδ is an inhibitor of the phosphorylated form of protein kinase B (Akt) in primary keratinocytes [170]. However, in cancer cells it has been shown to increase pAkt activity, which is important because phosphorylation of Akt plays a suppressive role towards apoptosis, in part by preserving mitochondrial integrity and ultimately

preventing the release of cytochrome c [171]. This has cardiovascular implication, because translocation of the phosphorylated form of Akt to mitochondria from the cytosol provided protection in late pre-conditioning via the mitochondrial ATP-sensitive K^+ channels (mitoK(ATP)) [172]. Angiopoietin-like 4 is a well-known target gene of PPAR activation [173]. It was recently shown that DHA was a potent activator of it (70-fold induction over control), as compared to decanoic, oleic, and lino oleic acid which were less potent [174], suggesting that DHA has the potential via PPARδ signaling to lead to profound changes in gene expression.

1.7 PPAR INTERACTION WITH n-3 & n-6 PUFAs

An emerging area of research is the interaction between n-3 PUFAs as ligands for PPARs. It has been shown that the three PPAR isoforms display differential sensitivity in terms of their potential for activation by fatty acids. Naruhn et al. demonstrated that the AA metabolites 15-hydroxyeicosatetranoic acid potently activated PPARδ, but only weakly influenced PPARγ and PPARα [173]. n-3 PUFAs (and their metabolites) via binding to nuclear receptors such as the PPARs can influence gene transcription, with this activation being due to a change in the 3-D structure of the receptor complex [118] [175]. Experimental evidence from 3T3-L1 adipocytes has demonstrated that a single 24 hour treatment of DHA (125 μM) can up-regulate PPARγ mRNA expression [176]. It is important to note that metabolites of n-3 PUFAs such as DHA may have increased potency as compared to their parent compound. As has been shown before, and well summarized by Neels and Grimaldi, high concentrations of fatty acids are typically required to activate PPARs due to their rapid metabolism. However, fatty acid derivatives and stabilized eicosanoids are active at lower concentrations, suggesting that metabolites may act more potently [116]. This is supported by a previously published paper showing that DHA derivatives were more readily available ligands for PPARγ activation [177]. Thus, it is becoming increasingly more recognized that fatty acid metabolites may be the key activators of the PPAR isoforms [116].

1.8 CERAMIDE

1.8.1 CERAMIDE BIOSYNTHESIS & BACKGROUND

Ceramide consists of a fatty acid bound to the amino group of sphingosine, making it a N-acylsphingosine [178]. Ceramides vary in size, containing between 2 and 28 carbon atoms in their N-fatty acyl chains, although chains of 16-24 carbons are most commonly represented in cell membranes [179] [180, 181, 178]. There is a ubiquitous ability for various cells and tissues to generate ceramide *de novo* [179]. One of the major well-characterized pathways for the generation of ceramide from less complex molecules is catalyzed by the enzyme serine palmitoyltransferase (SPT), which generates *de novo* ceramide via condensation of palmitoyl-CoA and L-serine at the ER [182, 178, 183] (figure 1.2). Another pathway for ceramide generation is directly at the cell membrane, where sphingomyelinases break down sphingomyelin [178, 184]. Finally, ceramide can be created via the salvage pathway, which constitutes the majority of sphingolipid biosynthesis by the breakdown of complex sphingolipids in acidic organelles such as lysosomes and late endosomes [178].

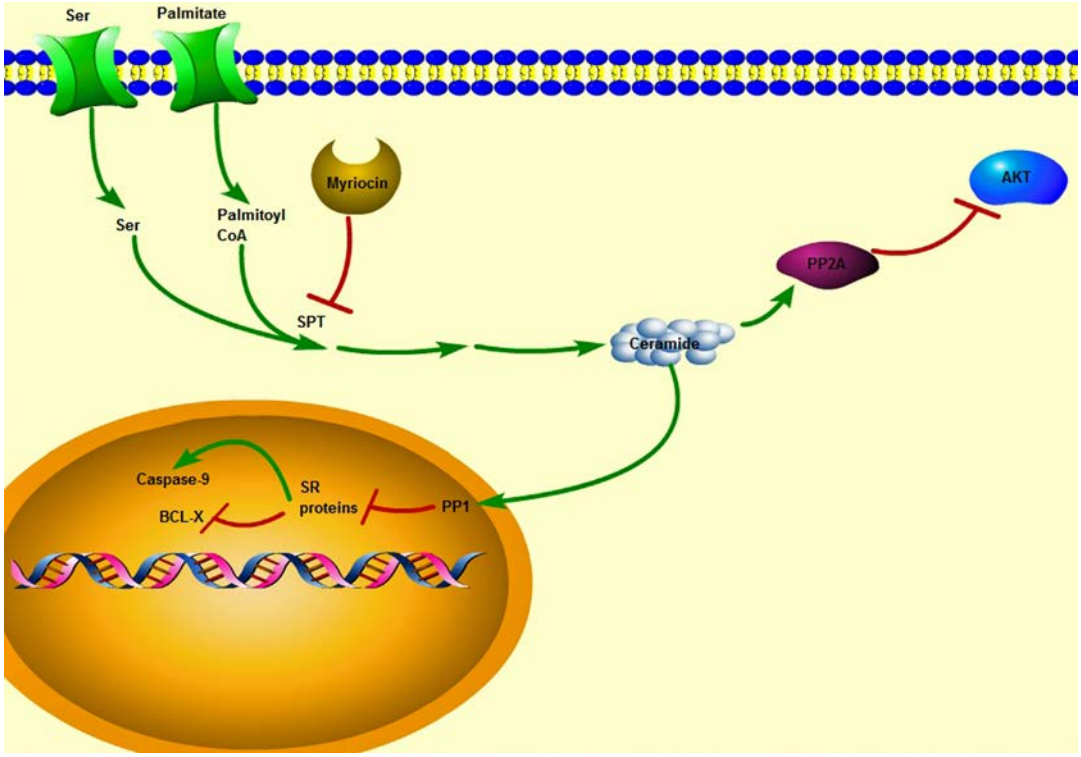

Fig 1.2 Pathway for *de novo* biosynthesis of ceramide (adapted from ref 183).

Serine is combined with the acylated form of palmitate (palmitoyl CoA) leading to sphingolipid synthesis. If excess levels are palmitate are present, this can result in *de novo* synthesis of ceramide. Ceramide is known to inhibit Akt signaling via activation of protein phosphatase-2A (PP2A). Nuclear activation of PP1 by ceramide leads to initiation of apoptosis, in part, by the activation of caspase-9.

1.8.2 CERAMIDE- MEDIATED SIGNALING & CELLULAR EFFECTS

Ceramide has been implicated as a mediator for a diverse array of cellular signaling cascades converging on such things as cell death, proliferation, and angiogenesis [185] [186], and is involved in cell death signaling in yeast [187], as well as in mice [188]. Consistent with other secondary messengers involved in cell signaling, ceramide fits this role due to its rapid and temporary creation, with the ability to affect many diverse cellular processes such as differentiation, inflammation, and apoptosis [181] [178]. Ceramide is part of the sphingomyelin pathway, which is evolutionarily conserved, and appears to have developed even before caspase-mediated cell death [189, 190].

Cardiolipin is a mitochondrial phospholipid that plays a role in maintaining functionality of various inner mitochondrial membrane enzymes, as well as in experimental heart failure [86, 191]. It is also interesting to note that a decrease in the amount of cardiolipin can increase ceramide accumulation via mitochondrial ceramidase, suggesting a potential role for ceramide in the functionality of the hearts [192]. Cells can also undergo apoptosis due to a lipid overload (such as ceramide), which is a relatively recently defined type of cell death referred to as lipoapoptosis. This typically occurs when non-adipose tissues accumulate long-chain FA [86]. In addition, an excessive amount of the lipid palmitoyl CoA eventually forms *de novo* ceramide inducing lipoapoptotic cell death in beta cells [193]. Lipoapoptosis has been attributed to a

pathophysiological role in beta cells [193], heart disease and heart failure [194 195], and cancer [196 197 198], suggesting that excessive formation of ceramide can lead to many diverse pathophysiologies.

1.8.3 ROLE OF CERAMIDE IN DISEASE

There is a growing abundance of literature adding to the well-defined connection suggesting that the accumulation of ceramide plays an intimate role in apoptotic signaling [199 200 201 202 203 204 187]. Ceramide has previously been shown to be involved in the 'initiation' phase, prior to the 'effector' phase of apoptosis, thus occurring at a relatively early time point. However, these results were obtained in HeLa cells, and other cell lines may have varying time-dependent signaling of cell death [205]. In addition, via breakdown of plasma membrane sphingomyelin, ceramide can be generated and influence formation of apoptotic bodies during the effector phase [206]. Many of the morphological alterations that occur during apoptosis happen as the result of activating caspases that function as a class of intracellular proteases [88]. All caspases are cysteine proteases which cleave their substrates at aspartate residues [207]. In order to avoid unwarranted cell death, caspases are kept in their inactive forms, which have a pro-domain and two subunits along with a catalytic domain [207, 208]. Once activated, caspases operate in a hierarchical manner, which allows for the amplification of apoptotic signals [209, 210]. In terms of hierarchy and time-course,

increases in the generation of ceramide typically occur relatively early in apoptosis before the activation of caspase 3, highlighted by the fact that ceramide accumulates independently of the effector caspases [211] [212] [213].

One important consequence of too much generation of ceramide is that if it is accumulated within mitochondria, this leads to the eventual release of intermembrane space proteins such as cytochrome c into the cytosol, thus initiating the well-characterized pathway of apoptosis resulting from this [180] [214] [215] [216, 217] [218]. Interestingly, this ceramide-induced loss of mitochondrial potential and cell death may be caused by direct insertion of columns of ceramide monomer aggregates, which form highly ordered barrel-stave channel(s) within phospholipid membranes allowing proteins to escape [180] [219] [220] [221]. Control of the channel has not been fully elucidated, but Bcl-2 proteins such as Bax and Bcl-xL play a role [222], and this channel can span as far as the mitochondrial outer membrane permeabilization (MOMP) leading to a reversible increase in permeability to cytochrome c [180]. Excess mitochondrial ceramide can also cause MOMP by inducing Bax insertion within the outer mitochondrial membrane [223]. Furthermore, the mole fraction of ceramide itself when measured during early apoptosis was enough to induce significant permeabilization of the MOMP [180]. Although clinical results have been mixed, administration of EPA and DHA ethyl esters for 2-3 weeks before cardiac surgery can be beneficial, as this has been correlated with an improvement in mitochondrial respiration [224]. Moreover, accumulation of C_2 ceramide has been

linked to a time and concentration-dependent mitochondrial network fragmentation (i.e. fission), which contributed to apoptosis of cardiomyocytes [225]. This study was important because it was the first to show that an accumulation of ceramide may be mechanistically involved in cardiomyocyte apoptosis due to mitochondrial fission [225], thus highlighting the potentially detrimental role of ceramide within the heart, along with its previously established role in tumorigenesis, with the prerequisite of MOMP occurrence for apoptosis possibly tying the processes of cardiomyocyte death and tumorigenesis, with MOMP being more likely to occur in areas undergoing mitochondrial fission [226]. In addition, there is a close biophysical association due to proximity to one another between the MOM and *de novo* synthesized ceramide at the ER [227] [228]. The formation of ceramide channels can be ascertained by changes in membrane permeability, which leads to an increased flow rate for solutes/ions [180]. It is interesting to note that ceramides have also been detected in the mitochondrial associated membrane, suggesting that in addition to the ER, mitochondria are an important organelle in the regulation of ceramide metabolism and synthesis [179]. It is also interesting to note that pro-apoptotic proteins such as Bax can become localized to, and act in a synergistic fashion adding stability together with ceramide in the formation of these channels [180] [229]. An interesting study showed that the drug SDZ PSZ 833 may induce its chemosensitizing affect in a diverse array of breast cancer cells via up-regulation of ceramide formation, which was preceded by increased levels of SPT activation [230].

Ceramide is commonly referred to as a tumor suppressive lipid [178], and increased ceramide synthase activity was implicated in nilotinib-mediated apoptosis in human chronic myeloid leukemia (CML) cells [231]. Increases in C16 ceramide has been shown to induce apoptosis in HeLa cells [232]. On the other hand, *de novo* generated C16 ceramide promoted survival in response to ER stress in squamous cell carcinoma [233]. A resveratrol-mediated synergistic apoptotic affect between C8-ceramide and a sphingosine kinase-1 inhibitor was demonstrated in K562 CML cells, suggesting that ceramides have the potential to be used an adjuvant cancer therapy [186]. Ceramide-induced target proteins are known to affect Akt and protein kinase C [179]. It has also been shown that C6-ceramide accumulation when co-administered with histone deacetylase inhibitors can significantly increase apoptosis in cancer cells by activating protein phosphatase 1, which then dephosphorylates Akt. This affect was also replicated in the same study *in vivo* using mice xenograft pancreatic and ovarian cancers [234]. Although we demonstrate that an increase in *de novo* ceramide biosynthesis induced apoptosis in our immortalized cell line, a balance in the amount of ceramide ultimately needs to exist, because it is also vital for cellular homeostasis since its complete inhibition may also lead to cancer and liver toxicity [179]. Thus highlighting the intricacies and complexities that exist even for a singular type of ceramide, and suggests that caution should be taken when considering a blanket approach towards up or down-regulating ceramides.

1.9 THESIS OVERVIEW

1.9.1 RATIONALE

There is a growing amount of evidence demonstrating cardioprotective benefits of n-3 PUFAs such as EPA and DHA [235-237]. PUFAs can cause changes to gene expression profile and improve cellular function, and they possess anti-inflammatory and anti-atherogenic functions [238, 239]. Modification of these factors can eventually lead to clinically relevant benefits such as increases in systolic left ventricular function [240, 241], decreased platelet activation and aggregation [242, 243], and decreased mortality [244]. In addition to their well-documented cardioprotective role, n-3 PUFAs have demonstrated protective effects against cancer and tumorigenesis, *in vitro* [94, 245, 246], *in vivo* [94, 245, 247], and clinically [248-252].

Despite the evidence for protective benefits, the in-depth cellular and molecular signaling pathways mediating these effects remain largely unknown. n-3 PUFAs are known to act as ligands for various transcription factors such as PPARs [118, 175, 253, 254]. A variety of models demonstrate that downstream signaling events after PPAR activation induce the formation of toxic products and mediators such as ceramide and ROS, leading to context-dependent protection from, or the potentiation of apoptotic cell death [255-258]. It appears that the ability for PPARs to induce cell death is cell and context-dependent, and remains to be established. It is unclear whether DHA can act via PPARs to

induce *de novo* formation of ceramide leading to cellular death in immortalized cells. Previously, our laboratory demonstrated that DHA triggers differential cellular responses, which depends on the type of cell being exposed. Interestingly, DHA had no significant adverse effects towards primary neonatal cardiomyocytes, but was cytotoxic toward H9c2 cardiac cells, an immortalized cell line. The DHA mediated effects triggered loss of mitochondrial membrane potential and an increase in cellular stress leading to apoptotic cell death [259]. How DHA induced this cytotoxic response was unresolved. Therefore, the overarching aim of the current study is to elucidate the cellular signalling mechanism by which DHA induces cytotoxicity in H9c2 cells, and to identify the role that PPARδ signaling plays in association with *de novo* ceramide accumulation in the induction of apoptosis.

1.7.2 HYPOTHESIS

We hypothesize that DHA-mediated cell death in H9c2 cells occurs by activating PPARδ-mediated signaling, which triggers an increase in de novo biosynthesis of ceramide leading to the initiation of apoptosis.

1.7.3 OBJECTIVES

1) To investigate the mechanism(s) by which DHA induces cytotoxicity in the immortalized H9c2 cardiac cell line.

2) To assess and determine the role of PPARδ signaling in DHA-mediated cytotoxicity.

3) To investigate the role of CYP-derived metabolites of DHA, such as 19,20-EDP, in H9c2 apoptotic cell death.

1.7.4 SIGNIFICANCE

Cardiovascular diseases and cancer account for over half of all cause of morbidity and mortality [260], with cancer and heart disease ranking as the first and second most common causes of death in Canada [261]. It is thus imperative for scientists and clinicians alike to elucidate the cellular and molecular signaling mechanism involved in both CVD and cancer, which can not only save lives, but can also mitigate enormous economic costs [262]. There has been significant progress made in understanding n-3 PUFA-mediated cardioprotection since the classic Greenland Inuit studies, and positive experimental and clinical links between n-3 PUFAs and protection against various cancers. However, these results have at times been inconclusive and inconsistent due to factors such as a patients age, species-specific differences, and previous history of disease [70, 263]. Our end-goal is to progress knowledge of the roles that n-3 PUFAs can have as protective mediators towards both CVD and cancer, and to find novel cellular pathways and mechanisms to achieve this.

We were particularly interested in PPARδ-mediated signaling, as compared to PPARα or PPARγ, because it is the least studied isoform [95, 264]. In addition, PPARδ is abundantly expressed within the heart, and is the dominant isoform within H9c2 cells [154, 265, 266]. The evidence in this thesis suggests that DHA can act in immortalized H9c2 cells through a previously unexplored pathway via PPARδ-mediated signaling. This has importance because as demonstrated, stimulation of the PPARδ receptor induces apoptosis. Furthermore, we go on to reveal a role for epoxy metabolites of DHA, such as 19,20-EDP in ceramide-induced cell death. This can open the door for new opportunities utilizing these metabolites, and can potentially stimulate future research and avenues employing them in animal studies, as well as in forthcoming drug design.

CHAPTER 2

PPARδ Signaling Mediates the Cytotoxicity of DHA in H9c2 Cells

#A version of this chapter has previously been published: Samokhvalov V, Zlobine I, Jamieson KL, Jurasz P, Chen CB, Lee KSS, Hammock DB, and Seubert JM. PPARδ Signaling Mediates the Cytotoxicity of DHA in H9c2 Cells. *Toxicology Letters* 232: 10-20, 2015.

1. INTRODUCTION

PUFAs such as docosahexaenoic acid DHA and EPA are metabolites of α-linolenic acid essential to cell survival. PUFAs participate in regulating many functions both at the cellular and systemic level [267]. For example, they are important components of cell membranes affecting their fluidity, composition and structure [268, 269]. Numerous studies report a pronounced positive effect of DHA towards the cardiovascular system [270], brain development [271], prevention of dementia and major depressive episodes [272]. Evidence demonstrates that DHA can produce effects in cancer and immortalized cell lines [273, 274], such as increased apoptosis, and has been shown to have anti-cancer properties *in vivo* [66]. Previously, we demonstrated that DHA triggered a concentration-dependent death of the immortalized H9c2 cardiac cells but not in primary neonatal cardiomyocytes [259]. The fundamental difference between immortalized and primary cardiac cells dictates the importance of obtaining a better understanding of DHA-mediated biological events, which is important for the development of DHA-based therapeutic strategies to treat various pathogenic conditions.

DHA has been shown to initiate a cytotoxic response in some cancer cells through(PPARs [275]. PPARs are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily [120, 276]. PPARs function as nuclear receptors to various molecules, including lipid mediators, regulating cellular functions associated with fatty acid metabolism [140, 277] and lipid

transport [123]. Additionally, PPARs have a role in inflammatory responses [132, 277], cell differentiation [278] and tissue development [279]. There are three PPAR isotypes described (α, γ, and β/δ) regulating physiologically distinct processes [265]. PPARα is pro-carcinogenic in hepatocytes, while PPARγ activity leads to terminal differentiation in prostate and breast cancer cells, as well as a reduction in infarct size in hearts [123, 280].

PPARs are involved in regulating numerous pathways impacting both physiological and pathophysiological conditions such as cardiovascular disease and cancer. Activation of PPARs can result in intracellular accumulation of ceramide, which mediates downstream effects such as apoptosis [281, 282]. DHA has previously been shown to increase the formation of ceramide *in vitro* [283]. In addition, DHA acts as a ligand for PPARs [284]. The role of PPARδ in carcinogenesis appears to be the least studied and the most controversial [264, 285]. PPARδ is ubiquitously expressed in all adult tissues including the heart [154, 265]. Given the physiological proximity of immortalized cell lines with cancer cells and the fact that H9c2 cells are known to abundantly express the PPARδ isoform [266], our objective in this study was to elucidate the role of PPARδ signaling associated with ceramide accumulation in DHA-induced cytotoxicity.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

DHA (cat # 90310), *N*-methylsulphonyl-6-(2-proparglyloxyphenyl) hexanamide (MS-PPOH) (cat # 75770) and 19, 20 – EDP (cat # 10175) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Tissue culture materials were obtained from Invitrogen (Burlington, ON, Canada); bradford protein assay solution from Bio-Rad Laboratories (Mississauga, ON, Canada); primary and secondary antibodies from Cell Signaling (Pickering, ON, Canada); trypan blue, MTT, 2', 7'-Dichlorofluorescin diacetate, myriocin from Sigma-Aldrich (Oakville, ON, Canada). Annexin V-FITC and propidium iodide from BD sciences; caspase 3 assay kit from Sigma (Saint Louis, MO, USA cat# CASPC3C-1KT); total 20S proteasome assay kit from Chemicon (EMD Millipore, Etobicoke, ON, Canada cat # APT280); lipid peroxidation (MDA) (cat # ab118970); PPARδ transcription factor assay kit Cayman Chemicals (Ann Arbor, MI, USA cat # 90310). GSK -3787 (cat# 3961); and Ceramide (cat # 0744) from Tocris Bioscience (Bristol, UK). Cell Counting Kit-8 (CCK-8) (cat# CK04-05) from Dojindo Molecular Technologies Inc. (Burlington, ON, Canada), EDP methyl esters regioisomer mixtures (1:1:1:1:1:1; 4,5-, 7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP) were prepared as described previously (Morisseau et al, 2010).

2.2. Cell culture

H9c2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and streptomycin at 37 °C in an atmosphere of (5% CO_2/ 95% air). H9c2 cells were plated at 25-30% confluency and cultured until they reached 75-80% confluency, upon which time they were treated with 0 or 100 μM DHA, and then harvested and subjected to various assays. During some experiments cells were treated in the presence or absence of the selective PPARδ antagonist (GSK 3787, (Tocris Bioscience, Bristol, UK). GSK 3787 was used at a concentration of 1 μM based on previously established data [286]. DHA was used at a concentration of 100 μM dissolved in ethanol, which was based on our previous study demonstrating that DHA has a concentration-dependent response triggering cytotoxicity in H9c2 cells [259]. Control groups were administered vehicle solution (<5% ethanol, ddH_20).

2.3. Western blotting

Samples were subjected to Western blot analysis as previously described [259]. Briefly, H9c2 cell samples (20 μg protein) were resolved on 12% SDS-polyacrylamide gel and then transferred electrophoretically to nitrocellulose membranes, that were blocked in TBS-T buffer (0.15 M NaCl, 3 mM KCl, 25 mM tris hydroxymethyl methylamine and 0.1% tween-25, pH 7.4) with 5%

skim milk for 2 h at room temperature. Membranes were washed four times with TBS-T buffer, and then incubated with a phospho-Akt antibody for Ser473 (1:500, Cell Signaling, cat #9271) overnight at 4°C. Membranes were washed as described above and then incubated with horseradish-peroxidase linked anti-rabbit IgG secondary antibody (1:10,000, Cell Signaling, cat #7074) for 2 h at room temperature. A similar procedure was used to probe membranes for total Akt (1:1000, Cell Signaling, cat #9272S), and we utilized GAPDH (1:1000, Cell Signaling, cat #2118S) and ULK-1 (1:1000, Cell Signaling, cat #8054) as loading controls. Chemiluminscence solution was used to detect signals. Relative band intensity was expressed in arbitrary units, and was assessed by densitometry using Image J software (NIH, Bethesda, MD, USA).

2.4. Cell viability and metabolic activity

Cell viability was estimated based on a modified Trypan blue exclusion method as previously described [259]. Briefly, 0.4% of Trypan blue solution was added to cells for 15 min at room temperature. Cells were then centrifuged at 1000 x g for 5 min at room temperature. The supernatant was gently aspirated and cells were washed three times with ice-cold PBS. 1% sodium dodecyl sulfate solution was added to lysed cells. Optical density was read spectrophotometrically at 595 nm. In a separate set of experiments, CCK-8 was utilized as a measure of cellular viability [287, 288]. This assay utilizes the water-soluble tetrazolium salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8). Colorimetric changes representing

cellular viability are assessed by the reduction of WST-8 via cellular NADH dehydrogenases, with 1-Methoxy PMS acting as an electron mediator, ultimately producing an orange colored formazan dye. This color is representative of cellular dehydrogenase activities, and correlated with the amount of viable cells.. CCK-8 was added to H9c2 cells in 96-well plates, and incubated in the dark for 2 hours. Optical density was read spectrophotometrically at 450 nm. Cellular activity was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) to formazan crystals as described previously [259]. The MTT assay is a widely used colorimetric assay for estimation of cellular activity [289]. Briefly, MTT solution was added to incubated cells for 6 h at 37◦C prior to assessment. Optical density was measured spectrophotometrically at 595 nm and the cellular activity ratio was represented relative to control. Release of lactate dehydrogenase (LDH) was measured colorimetrically using a modified MTT protocol as described by Abe and Matsuki [290].

20S total proteasome activity was measured using an assay kit (EMD Millipore, Billerica, MA) based on the detection of 7-amino-4-methylcoumarin (AMC) fluorescence in cell lysates after cleavage of the peptide LLVY-AMC. Fluorescence was measured at 380 nm excitation and 460 nm emission. A standard curve was established with AMC. Caspase-3 was assessed as described previously [259], using the selective substrate Ac-DEVD-AMC. Briefly, caspase-3 activity was determined in cell lysates by detection of AMC fluorescence after cleavage of the peptide. The fluorescence was monitored at 460 nm and excited

at 380 nm. The activity was calculated by using a linear standard curve found with AMC.

2.5. PPARδ DNA binding activity

PPARδ DNA binding activity was measured in cell lysates by ELISA assay Cayman Chemicals (Ann Arbor, MI, USA cat # 10006914), based on a double stranded DNA sequence containing the peroxisome proliferator response element (PPRE). Activated PPARδ present in the cell lysates binds to the PPRE and is detected via primary and secondary antibodies.

2.6. Markers of Oxidative Stress

Lipid peroxidation was assessed in cell lysates (Abcam Inc., Toronto, ON), based on measurement of the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) fluorometrically at 553 nm (emission) and excited at 532 nm. ROS generation was measured using the cell permeable probe 2', 7'-Dichlorofluorescin diacetate (H_2DCF-DA) that is converted to the fluorescent product 2', 7'- dichlorofluorescein upon removal of its diacetate groups via intracellular esterases, which allows for the overall quantification of intracellular redox status [291]. H9c2 cells were seeded in 24-well plates, and the production of ROS was determined after 6, 12, and 24 h of DHA treatment (0, 1, 10, 100 μM) by incubating cells with 30 μM of H_2DCF-DA in pre-warmed phenol-free Hank's buffer for 1 h (in the dark). Following this, the media was removed and replaced with pre-warmed phenol red-free Hank's buffer on its

own, and positive controls were treated with 100 μM of H_2O_2 for 30 min. Changes in fluorescence were recorded using a fluorescence plate reader (excitation 480 nm, emission 520 nm). Total antioxidant capacity (TAC) was measured using an Antioxidant Assay Kit (Sigma-Aldrich, Co, Oakville, ON). The specific assay is based on the formation of a ferryl myoglobin radical from myoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation $ABTS^+$, a soluble green color chromogen that was determined spectrophotometrically at 405 nm. In the presence of antioxidants the radical cation is suppressed to an extent dependent on the activity of the antioxidant and the color intensity is decreased proportionally. The water soluble vitamin E analogue trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a positive control antioxidant [292].

2.7. Flow Cytometric Detection of Apoptosis

Flow cytometry was employed to detect apoptosis as previously described [293]. In brief, trypsinized cells were washed with binding buffer and centrifuged at a low speed (220*g*). Following this, binding buffer was used to resuspend the cells with Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI), followed by incubation in the dark for 15min, and finally, diluted to 500μl. Fluorescence was induced with a 488-nm argon laser and detected on FL1 (525 nm BP filter), and FL3 (620 nm SP filter) on a

Beckman Coulter Cytomics FC500 flow cytometer (10,000 events per sample). Spectral compensation was performed using Cell Lab Quanta analysis software. Percent apoptotic cells were assessed by combining events falling in the lower and upper right quadrants (early and late apoptotic cells, respectively).

2.9 LC/MS Measurement of Ceramide

The extraction of ceramides from H9c2 cells was performed in accordance with a previously published protocol [294]. Cells were centrifuged at 500 g for 5 min then rinsed with cold PBS and centrifuged again at 500 g for 5 min. 300 μL of a 0.4% NaCl solution and 1 mL of a chloroform-methanol-1N HCl (100:100:1, v/v/v) mixture were added to the samples. Following this the samples were wrapped in parafilm and vortexed at 1000 rpm (at room temperature) for 20 min. Samples were then centrifuged at 20,000 g for 2 min, and the resulting organic phase was separated out and transferred into new vials. The samples were then evaporated using a Savant DNA 120 SpeedVac Concentration system (Thermo Fisher). The resulting pellets were then dispersed in the chloroform-methanol-HCl solution, and combined with 1μg/mL of freshly prepared yohimbine hydrochloride, which was used as an internal standard.

The samples were resolved using liquid chromatography-mass spectrometry (LC/MS) analysis, comprised of a Waters ZQ 4000 Mass

Spectrometer coupled to a Waters 2795 Separations Module (LC + autosampler). Ceramides were detected using a single ion recording in electrostatic ionization mode on a Waters Xterra C18 column (2.1 x 50mm, 3 μm). The parent ions were observed at m/z = 343.2 and 355.2 for ceramide and yohimbine, respectively. An A:B:C gradient system (0.225 mL/min) was used composed of acetonitrile (containing 0.005% acetic acid), water containing 0.2% NH_4OH and 0.05% NH_4OAc, and methanol. During the acquisition of the data, the mass spectrometer was maintained at a source and desolvation temperature of 140 °C and 250 °C, and a cone voltage of 20 V and 24 V, respectively. The resulting values were then calculated using a ceramide standard curve and expressed as mg weight per mg of cellular mass, and then further normalized to account for the fold change between wet and dry cellular mass for quantification purposes.

2.10. *Statistical analysis*

Values are expressed as mean ± SEM. Statistical significance was determined by the use of ANOVA. To determine whether significant differences exist between the groups, a Bonferonni post hoc test was performed. Values are considered significant if $p < 0.05$.

3. RESULTS

3.1. Treatment with DHA results in a dramatic decline in cell viability and cellular metabolic activity via PPARδ-signaling.

We demonstrated that treatment with DHA (100 μM) induced a pronounced decrease in cell viability as assessed by Trypan Blue exclusion (Fig 1A), which is consistent with our previously published study [259]. The deleterious effects of DHA were attenuated by co-treatment with the selective PPARδ antagonist, GSK 3787 (1 μM, Fig. 2.1A). Further evidence of DHA-induced cytotoxicity was observed by a decrease in cellular metabolic activity, based on detection of the reduced form of MTT. Figure 1B shows that DHA progressively lowered cellular metabolic activity in H9c2 cells over the 48h time course of exposure. Similarly, addition of GSK 3787 reduced the cytotoxic effect of DHA toward cellular metabolic activity in H9c2 cells. Assessment of the release of LDH from H9c2 cells exposed to DHA provided more evidence of a cytotoxic effect. DHA triggered a large release of LDH from cells within 24h, which was attenuated by co-treatment with GSK 3787 (Fig. 2.1C). Total proteasome activity reveals intracellular accumulation of ubiquinated proteins, thus representing a marker of unspecific cellular degenerative processes. Figure 2.1D demonstrates that treatment with DHA robustly increased total proteasome activity in H9c2 cells, which was suppressed by co-treatment with GSK 3787.

Overall, DHA-induced cytotoxicity was greatly attenuated by a selective inhibitor of PPARδ suggesting there was an involvement of PPARδ-signaling. In order to further validate the role of PPARδ signaling in mediating DHA cytotoxicity, we measured PPARδ DNA binding activity in H9c2 cells exposed to DHA with or without GSK 3787. This assay revealed that treatment with DHA for 24h resulted in a marked increase in PPARδ DNA binding activity. As expected, co-treatment with GSK 3787 prevented DHA-induced activation of PPARδ DNA binding activity (Fig. 2.1E). These results suggest that DHA-induced cytotoxicity involves activation of PPARδ signaling in H9c2 cells.

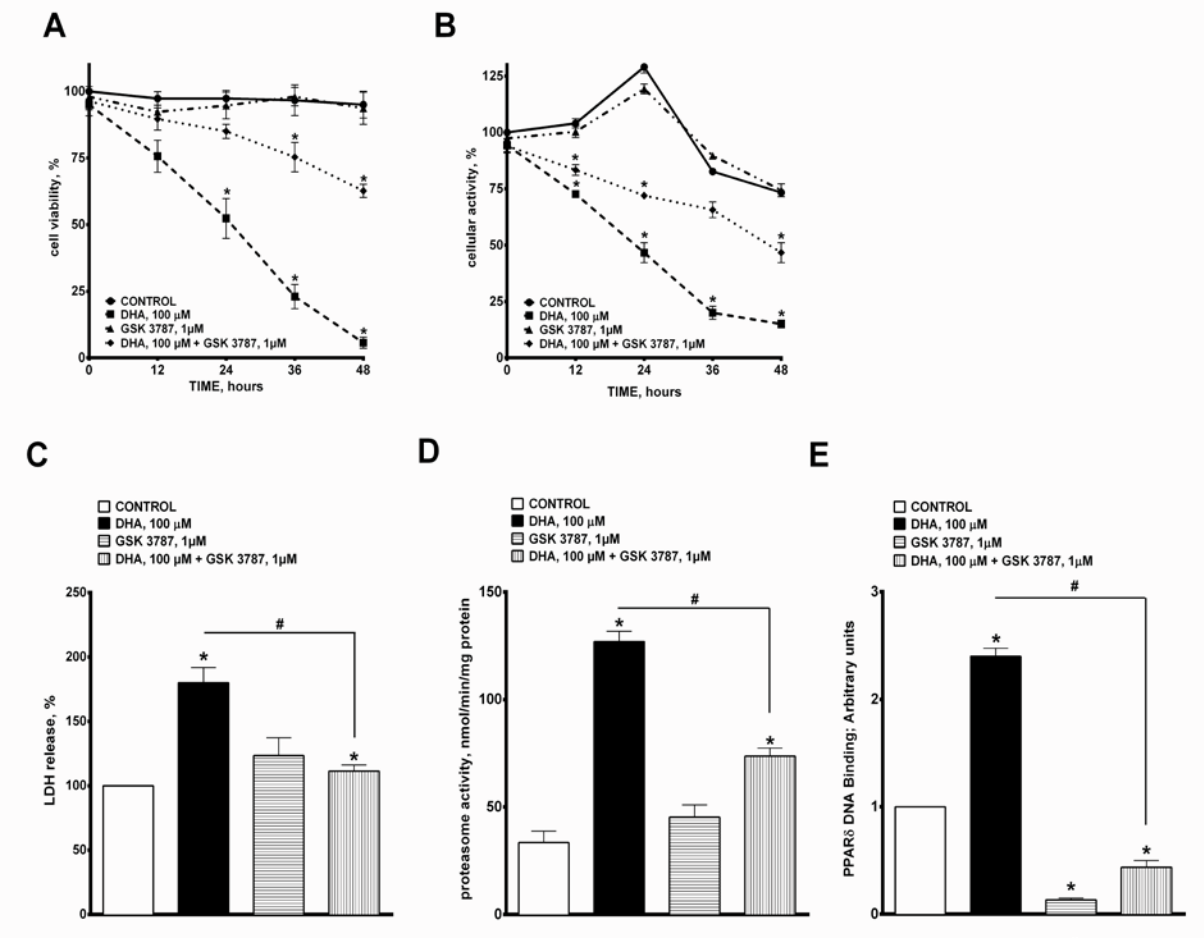

Figure 2.1: Effect of DHA exposure on cellular viability and metabolic activity. (A) Cell viability was estimated based on the Trypan Blue exclusion method. H9c2 cells were treated with 0 or 100 μM of DHA, and assessed at 24h. (B) Cellular metabolic activity was estimated by the reduction of MTT to formazan. (C) The percentage of LDH release was quantified colorimetrically. (D) Proteasomal activity was assessed by the detection of 7-amino-4-methylcourmarin (AMC) in cell lysates post cleavage from the peptide LLVY-AMC. (E) PPARδ DNA binding was quantified using an ELISA kit. Values

are expressed as mean ± SEM; N=3; *, $p<0.05$ DHA 100 μM vs. control, #, $p<0.05$ DHA 100 μM vs. DHA 100 μM and GSK 3787 1 μM.

3.2. Treatment with DHA has no effect on oxidative stress in H9c2 cells.

To examine whether the DHA-induced cytotoxicity occurred via activation of a general stress response such as oxidative stress rather than through an involvement of selective molecular mechanisms we assessed ROS production and accumulation of TBA-reactive products (MDA and 4-HNE) in H9c2 cells. We found that H9c2 cells treated with H_2O_2 (100 μM) induced a ~9-fold increase in ROS production (data not shown) while DHA treatment triggered a mild but non-significant increase in ROS (~1.3-fold) (Fig. 2.2A, and Fig. 2.2B displaying changes in TBA-reactive products). As endogenous antioxidant capacity of cells is an important component in understanding cellular responses to stress, we next assessed the effect of DHA on TAC in H9c2 cells. Interestingly, DHA treatment significantly depleted the antioxidant capacity in H9c2 cells after 24h (Fig. 2.2C). This effect was attenuated by co-treatment with GSK 3787 (Fig 2.2C). These data suggest that DHA triggered ROS production was neutralized by the endogenous antioxidant system in H9c2 cells. Moreover, the DHA effect specifically involved PPARδ signaling and the contribution of unspecific stress reactions in DHA-induced cytotoxicity was not significant.

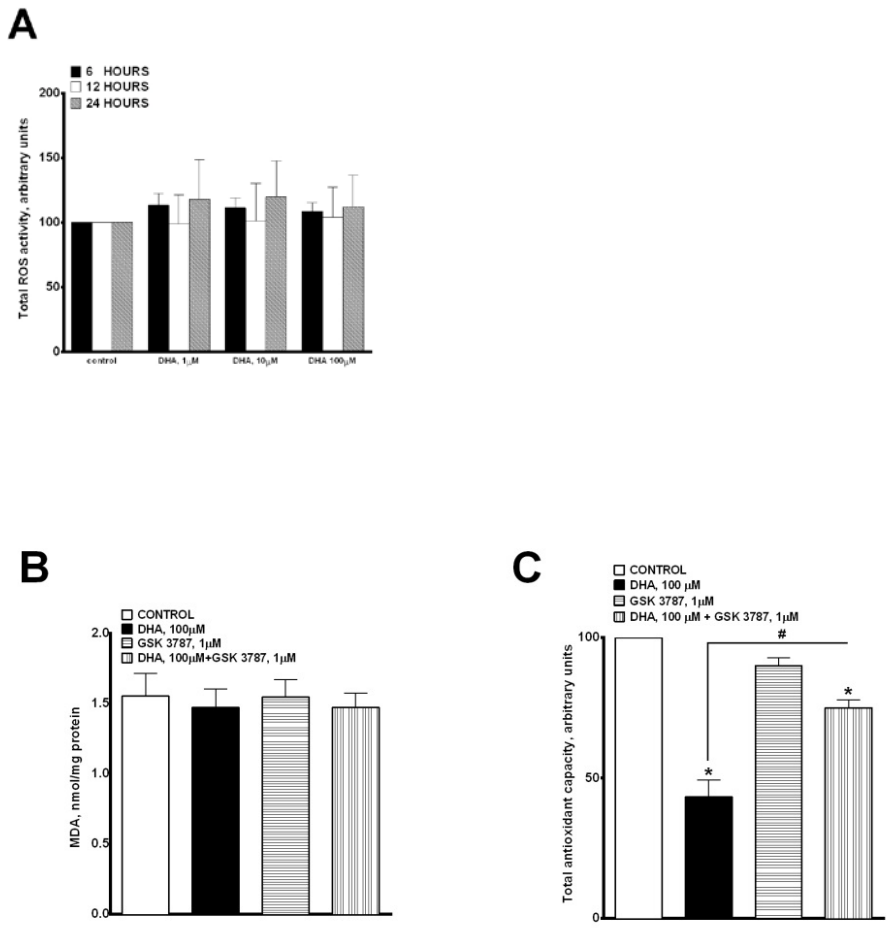

Figure 2.2: Effect of DHA on oxidative stress. (A) Histogram representing the generation of reactive oxygen species in H9c2 cells treated with 0, 1, 10 or 100 μM of DHA at 6, 12 and 24 hours. (B) Lipid peroxidation was determined by the accumulation of TBA-active products after 24h of treatment. H9c2 cells treated with 0 or 100 μM of DHA with and without GSK3787 (1 μM), a PPARδ antagonist. (C) Total antioxidant capacity was measured in H9c2 cells following 24h treatment with 0 or 100μM of DHA with and without GSK 3787 (1 μM). Values are expressed as mean ± SEM; N=3; *, $p<0.05$ DHA 100 μM vs. control, #, $p<0.05$ DHA 100 μM vs. DHA 100 μM and GSK 3787 1 μM.

3.3. DHA induces apoptosis in H9c2 cells mediated via PPARδ-signaling.

DHA-mediated cytotoxicity is believed to involve a complex interplay of numerous cell death/survival pathways. In this part of our study we estimated the overall apoptotic response based on the measurement of Annexin-V FITC binding, phosphorylation of Akt, and caspase-3 activity in H9c2 cells exposed to DHA. We implemented flow cytometric analysis utilizing Annexin-V FITC and propidium iodide (PI) to further examine the mechanisms of DHA-mediated apoptosis. Annexin-V staining is vital because it provides accurate detection of both early and late apoptosis [295]. DHA treatment led to a large increase in apoptosis (Figs. 2.3A-C). Interestingly, co-incubation of DHA with GSK 3787 led to a significant attenuation of the apoptotic response induced by DHA treatment (Fig 2.3D). The specific values for all four treatment conditions are summarized in Fig 2.3E.

In order to further validate the apoptotic response we examined if treatment with DHA affected phosphorylation of Akt, a key component of intracellular anti-apoptotic mechanisms [296]. We demonstrated that 24h of exposure to DHA resulted in reduction of Akt phosphorylation in H9c2 cells (Fig. 2.3F). This corresponded with a dramatic increase in caspase-3 activity (Fig. 2.3G), strongly suggesting activation of apoptosis. Co-treatment with GSK 3787 prevented the loss of phosphorylated Akt and reduced caspase-3 activity.

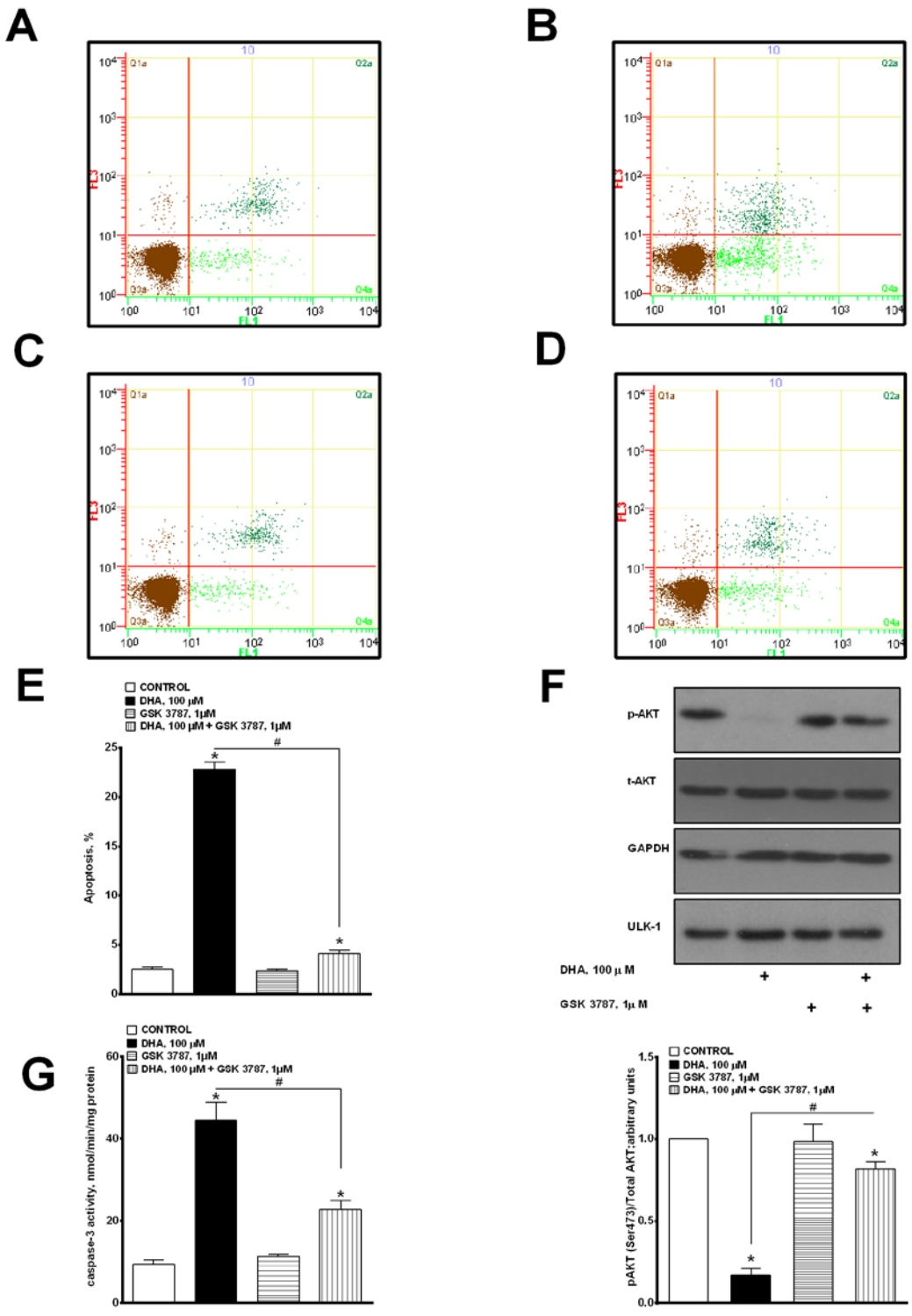

Figure 2.3: DHA-induced apoptosis mediated via PPARδ-signaling. Representative bivariate dot plots of Annexin-V FITC and propidium iodide

binding (A, control; B, DHA 100 μM; C, GSK 3787 1 μM; D, DHA 100 μM + GSK 3787 1 μM). Apoptotic cells were assessed by combining values falling in the upper and lower right quadrants. (E) Histogram summarizing flow cytometry data demonstrating induction of DHA-induced apoptosis (early and late combined). (F) Cells were harvested and subjected to western blotting to assay the level of p-AKT (upper insert), and quantified (lower insert). (G) Caspase-3 activity was assayed fluorometrically using the substrate Ac-DEVD-AMC. Values are expressed as mean ± SEM; N=3; *, $p<0.05$ DHA 100 μM vs. control, #, $p<0.05$ DHA 100 μM vs. DHA 100 μM and GSK 3787 1 μM.

3.4. Pharmacological inhibition of de novo ceramide synthesis attenuates DHA-induced cytotoxicity.

Accumulation of ceramide has been linked to major perturbations in cell metabolism resulting in apoptotic cell death [281], and evidence suggests that DHA may increase the level of intracellular ceramide [283]. Furthermore, activation of PPAR-mediated signaling, particularly via PPARδ, can also enhance accumulation of ceramide in cells [282]. To determine the role of this pathway we treated cells with myriocin (1 μM), which is a potent inhibitor of SPT in order to block the *de novo* synthesis of ceramide. We demonstrate that co-treatment with myriocin attenuated DHA-mediated cell death (Fig. 2.4A), prevented DHA-triggered increase in caspase-3 (Fig. 2.4B) and total

proteasome activities (Fig 2.4C). Evidence of *de novo* ceramide synthesis was supported by the marked increase in ceramide levels following 24h treatment with DHA (Fig. 2.4D). Inhibition of PPARδ activation with GSK 3787 attenuated the DHA-mediated accumulation of ceramide (Fig 2.4D). These results support a suggestion that PPARδ plays a crucial role in DHA-mediated cytotoxicity associated with ceramide accumulation.

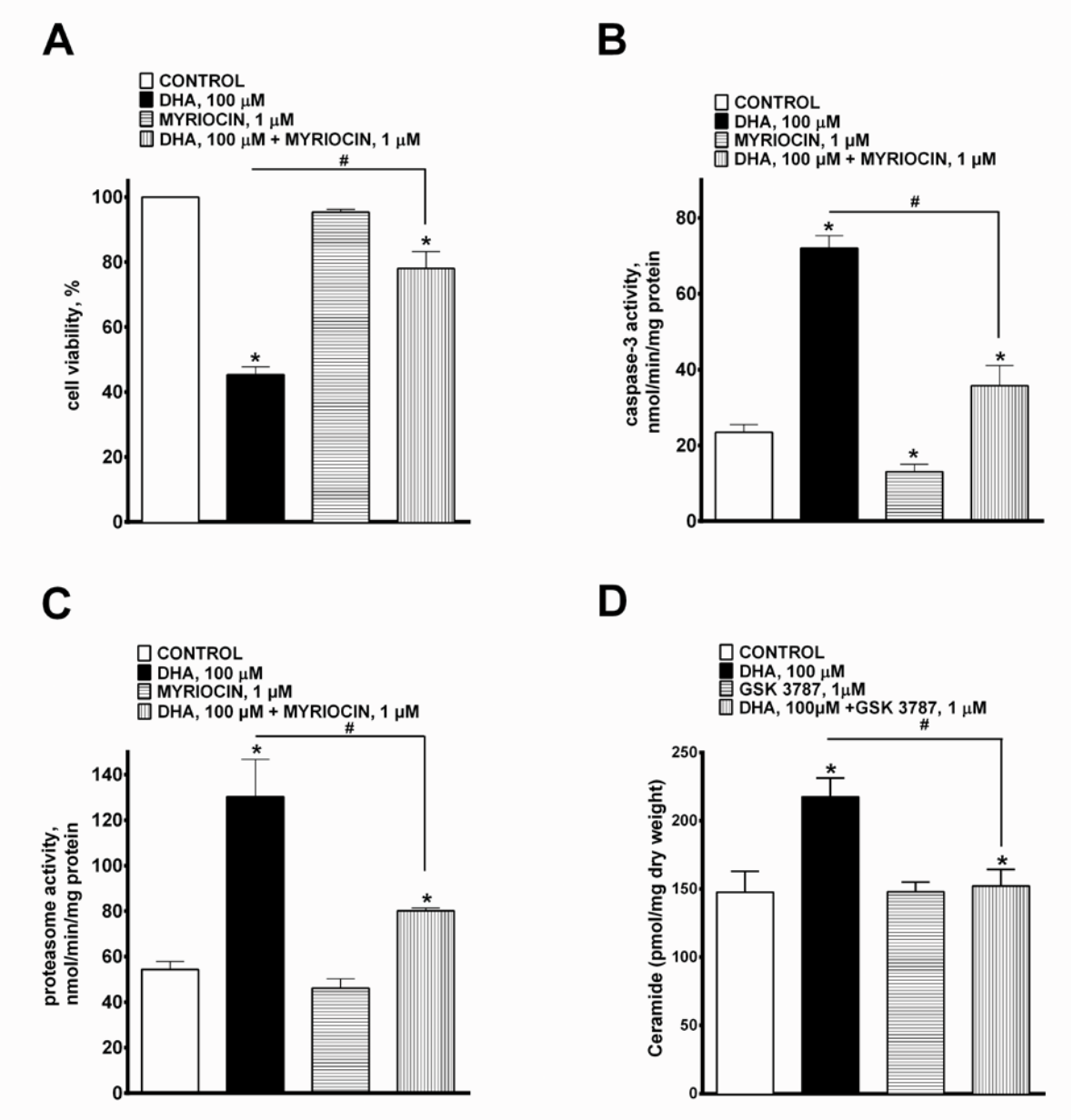

Figure 2.4: DHA-induced cytotoxicity is attenuated by pharmacological inhibition on *de novo* ceramide synthesis. H9c2 cells were treated with 0 or 100 μM of DHA with or without myriocin 1 μM, an inhibitor of SPT, and assessed at 24h. (A) Cell viability, (B) caspase-3 and (C) total proteasomal activities were measured. Co-treatment of DHA with myriocin significantly abolished DHA-induced cytotoxity. Values are expressed as mean ± SEM;

N=3; *, p<0.05 DHA 100 μM vs. control, #, p<0.05 DHA 100 μM vs. DHA 100 μM and myriocin 1 μM. (D) LC/MS analysis was employed to measure the accumulation of ceramide following treatment with 0 or 100 μM of DHA with or without GSK 3787 1 μM, a PPARδ antagonist, and assessed at 24h. Values are expressed as mean ± SEM; N=3; *, p<0.05 DHA 100 μM vs. control, #, p<0.05 DHA 100 μM vs. DHA 100 μM and GSK 3787 1 μM.

3.5. CYP oxidase metabolites of DHA, epoxydocosapentaenoic acids (EDPs), demonstrate cytotoxicity toward H9c2 cells.

Recent literature has suggested that cytochrome P450 oxidase metabolites of DHA, EDPs, act as autocrine and paracrine mediators to regulate cellular processes [42, 43]. In order to explore this notion, we performed experiments to investigate the role of EDPs in the DHA-mediated response. First, we treated H9c2 cells with DHA and/or MS-PPOH, an inhibitor of CYP oxidases, to determine if epoxide metabolites of DHA were involved in the cytotoxic response. Inhibition of CYP oxidase activity with MS-PPOH (50 μM) blocked DHA-induced cell loss and metabolic activity (Figs. 2.5A and B). Thus, we next tested the actions of EDPs using a mixture of six different EDP methyl ester regioisomers (1 μM) (1:1:1:1:1:1; 4,5-, 7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP methyl ester) or the individual isomer 19,20-EDP (1 μM) . Interestingly, both the mixture of EDPs and 19,20-EDP alone caused a

significant loss in cell viability and metabolic activity at a 100-fold lower concentration than DHA. The adverse effects of EDPs were attenuated by co-treatment with GSK 3787 (1μM) suggesting EDP-mediated events involved the activation of PPARδ (Figs. 2.5C and D). In order to confirm that EDP-mediated effects involved PPARδ, we performed a PPARδ DNA binding assay. We found that both the EDPs mixture and 19, 20-EDP alone increased PPARδ DNA binding activity, which was significantly diminished by co-treatment with GSK 3787 (Fig 5E). To further explore the signaling mechanisms, we assessed changes in ceramide production (Fig. 2.5F). The inhibition of CYP oxidases with MS-PPOH blocked the DHA-induced ceramide accumulation, while the EDPs mixture and 19,20-EDP alone triggered the accumulation of ceramide at a 100- fold lower concentration (Fig. 2.5F). The role of PPARδ in the EDP response was confirmed when GSK 3787 blocked ceramide production. These results support our hypothesis that the DHA metabolites, EDPs, specifically 19,20-EDP, induce cytotoxicity via a PPARδ-dependent pathway triggering ceramide production.

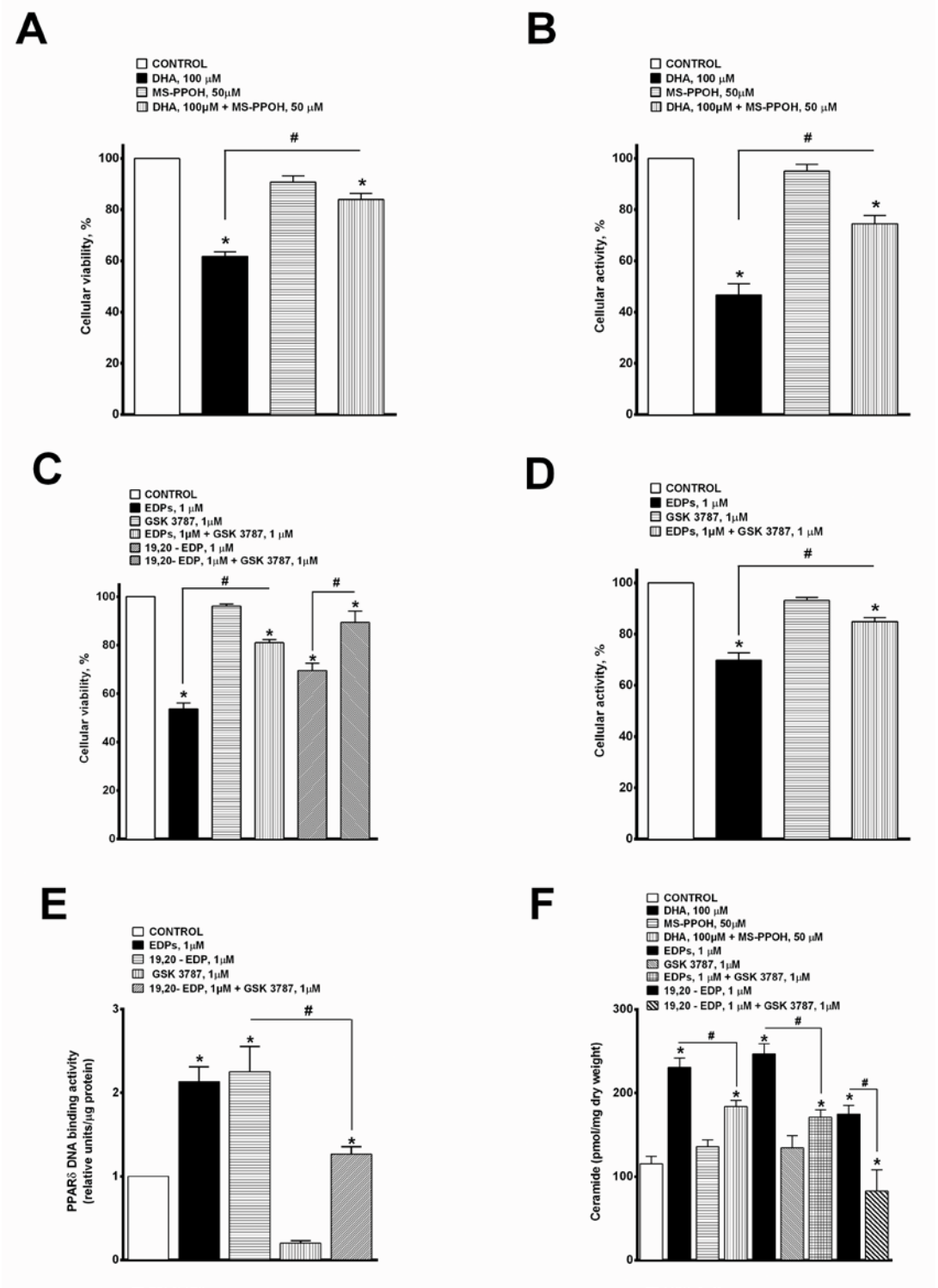
A
CONTROL
DHA, 100 μM
MS-PPOH, 50μM
DHA, 100μM + MS-PPOH, 50 μM
Cellular viability, %
B
Cellular activity, %
C
EDPs, 1 μM
GSK 3787, 1μM
EDPs, 1μM + GSK 3787, 1 μM
19,20 - EDP, 1 μM
19,20- EDP, 1μM + GSK 3787, 1μM
D
E
PPARδ DNA binding activity (relative units/μg protein)
F
Ceramide (pmol/mg dry weight)

Figure 2.5: EDPs demonstrate cytotoxicity toward H9c2 cells. H9c2 cells were treated with 0 or 100 μM of DHA with or without MS-PPOH 50 μM and assessed at 24h. (A) Cell viability was assessed using CCK-8, and (B) cellular metabolic activity was assessed via MTT. Values are expressed as mean ± SEM; N=3; *, $p<0.05$ DHA 100 μM vs. control, #, $p<0.05$ DHA 100 μM vs. DHA 100 μM and MS-PPOH 50 μM. H9c2 cells were treated with 0, 1 μM of EDP mix (1:1:1:1:1; 7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP) or 1 μM 19,20-EDP with or without GSK 3787 1 μM and assessed at 24h. (C) Cell viability and (D) cellular metabolic activity were measured. (E) PPARδ DNA binding was quantified using an ELISA kit. Values are expressed as mean ± SEM; N=3; *, $p<0.05$ EDPs or 19,20-EDP 1 μM vs. control, #, $p<0.05$ 19,20-EDP 1 μM vs. 19,20-EDP 1 μM and GSK 3787 1 μM. (F) LC/MS analysis was employed to measure the accumulation of ceramide following treatment with 0 or 100 μM of DHA with or without MS-PPOH 50 μM, EDPs mix 1 μM (1:1:1:1:1; 7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP) or 1 μM 19,20-EDP with or without GSK 3787 1 μM and assessed at 24h. Values are expressed as mean ± SEM; N=3; *, $p<0.05$ EDP 1μM vs. control, #, $p<0.05$ EDPs 1 μM vs. EDPs 1 μM and GSK 3787 1 μM, #, $p<0.05$ 19,20-EDP 1 μM vs. 19,20-EDP 1 μM and GSK 3787 1 μM.

4. DISCUSSION

Our results demonstrate a novel mechanism through which DHA and its epoxy metabolites, EDPs, trigger a cytotoxic response in immortalized H9c2 cells. We found that DHA- and EDP-induced cytotoxicity is mediated via PPARδ signaling resulting in *de novo* ceramide biosynthesis.

In our previous study we found that DHA produced a concentration-dependent cytotoxic effect in H9c2 cells [259]. Consistent with that previous study, we found that DHA induced a rapid decline in viability of H9c2 cells, which persisted up to 48h. Furthermore, the significant release of LDH into the cell culture medium indicated loss of membrane integrity due to DHA cytotoxicity [290, 297]. To further explore the type of injury we demonstrated that DHA treatment led to the activation of total proteasome activity, the ubiquitin-proteasome pathway involved in removal of irreversibly denatured proteins. Activation of total proteasome activity therefore appears to be a marker of the rate of overall protein degradation as well as turnover, providing an estimate of cell injury [273]. DHA has previously been shown to lead to an increase in ROS levels leading to oxidative damage of membranes and lipids [274, 298]. Considering PUFAs are susceptible to oxidation, this can trigger significant lipid peroxidation cascades leading to cell death [66, 298-300]. While evidence suggests that DHA-induced cytotoxicity can involve initiation of oxidative stress, our results demonstrated that neither ROS production nor lipid peroxidation were markedly increased in our experimental model. However, the decreased total

antioxidant capacity suggests the endogenous systems in H9c2 cells suppressed any significant increase in ROS production. Importantly, the data indicate that DHA cytotoxicity occurred through a more specific cell death signaling pathway in H9c2 cells, which involved PPAR signaling.

PPARs are involved in regulating a range of cellular processes including metabolism, cell growth and differentiation [301]. Interestingly, the involvement of PPARs in regulating carcinogenesis has a dichotomous role both in the prevention and initiation of tumor transformation of normal cells [302]. The contribution of PPARs in regulating cellular homeostasis is multifaceted, whereby modulation of PPAR functional activity leads to profound alterations in many pivotal cell functions [303, 304]. Evidence from the literature demonstrates that DHA activates PPARs, including PPARδ [305, 306]. Importantly, H9c2 cells are known to abundantly express the PPARδ isoform whereas the other isoforms are almost undetectable [266]. The fact that we were able to attenuate DHA-mediated events by inhibiting PPARδ, and DHA enhanced PPARδ DNA binding activity in H9c2 cells supports the notion that PPARδ signaling is involved in DHA-mediated cytotoxicity.

DHA has demonstrated unique characteristics, whereby depending upon the cell type it can promote either cell survival or cell death [307, 308]. Importantly, it remains unresolved how DHA triggers a selective cell survival or cell death pathway. In the current study using the immortalized H9c2 cells, DHA clearly induced cytotoxicity via activation of an apoptotic response. Flow

cytometry analyses demonstrated that DHA increased Annexin-V binding reflecting activation of apoptosis [66, 309]. Annexin-V is a Ca^{2+}- dependent protein that is known to bind to phosphatidylserine (PS) with high affinity on the outer leaflet of the plasma membrane surface of apoptotic cells. This phospholipid is typically present on the inner leaflet of the plasma membrane, but during early apoptosis PS is translocated to the outer leaflet [274, 295, 310]. Thus, translocation of PS as measured by Annexin-FITC binding, can be used as a marker for the phospholipid-binding protein Annexin V [311], and the presence of the negatively charged phosphatidylserine lipid flipped to the outside of the cellular membrane can signal a dead/ or dying cell to macrophages [144, 312]. It is therefore possible to distinguish between early apoptotic cells (Annexin V positive and PI negative), as opposed to cells in the late stages of apoptosis (positive for both Annexin-V and PI).

We further demonstrated that DHA caused marked effects causing decreased Akt phosphorylation and increased caspase-3 activity in H9c2 cells. Phosphorylation of Akt is known to promote cell survival and suppress apoptosis via downstream pathways such as phosphorylation of BAD [296, 313]. Our results provide evidence that PPARδ signaling is important for DHA-triggered apoptosis. However, research on the role of PPARδ signaling in apoptotic responses is unclear [277]. In contrast to our data demonstrating that PPARδ has a role in triggering apoptosis, previous reports show that PPARδ activation prevented apoptosis in endothelial progenitor cells [314]. While data in

keratinocytes demonstrates that PPARδ induces apoptotic signaling may occur via induction of terminal differentiation [315]. These results may be partially explained by differences in the expression profiles of PPAR isoforms between various cell types [316].

Ceramides are an important family of bioactive lipids composed of sphingosine and a fatty acid. Emerging evidence suggests they are involved in a variety of cellular processes ranging from cell proliferation to cell death and they are well-known activators of apoptosis [317, 318]. Ceramides can be generated by *de novo* synthesis, sphingomyelin hydrolysis or through the salvage pathway, all of which can be activated in response to various stimuli. In cancer cells endogenous levels of ceramide are typically low, but after treatment with chemotherapeutic drugs these levels are increased triggering cell death [317]. Whereas in the heart, excessive lipid and ceramide accumulation is involved in cardiac contractile dysfunction, increased oxidative stress, and cardiomyocyte apoptosis [194, 319, 320]. DHA can increase synthesis of ceramide via activation of SPT a key enzyme in *de novo* sphingolipid biosynthesis from palmitate [283]. Interestingly, PPAR receptors are involved in regulating lipid homeostasis, where agonist activation of PPARs in rat hearts was shown to increase SPT activity and ceramide levels [321]. In the current study, we inhibited DHA-mediated events by blocking PPARδ-induced ceramide production, as well as SPT activity, suggesting a novel pathway for DHA-induced apoptosis in H9c2 cells. Furthermore, our observation of increased accumulation of ceramide in

DHA-treated cells correlates with the loss of Akt phosphorylation, as ceramide has been reported to impede Akt phosphorylation [322].

Metabolism of n-3 PUFAs such as DHA, can occur by cyclooxygenase, lipoxygenases and cytochrome P450 oxidases to numerous metabolites with various characteristics including anti-inflammatory and anti-angiogenic properties [42, 45]. CYP oxidases can convert DHA to epoxydocosapentaenoic acids (EDPs) [323], which have demonstrated novel properties impacting angiogenesis, thrombosis and cancer (Jung et al., 2012; Zhang et al., 2013). EDPs have been suggested as the active mediator leading to protection against arrhythmias within the heart previously attributed to the actions of DHA [40, 324]. Although many DHA metabolites have been shown to be biologically active, 19,20-EDP is of particular interest due to its various pronounced biological effects, particularly toward the cardiovascular system [40, 48]. Inhibition of CYP oxidase activity with MS-PPOH has been shown to reduce the production of DHA-derived epoxy metabolites [48]. Experimental evidence indicates that the addition of MS-PPOH can inhibit the hyperactivity protective effects of the n-3 PUFA EPA, in a human bronchi model [325]. In our model, the simultaneous addition of MS-PPOH prevented the DHA-mediated cytotoxicity suggesting the involvement of CYP oxidase metabolites. The cytotoxic effect of EDPs, specifically 19,20-EDP, further supported the idea that the epoxy metabolites of DHA are active lipid mediators. Indeed, the fact that the EDP-mediated increase in ceramide levels were blocked by the PPARδ antagonist

demonstrates a novel mechanism of action. Collectively, these data highlight epoxy metabolites of DHA as important biologically active lipids regulating cellular function. Evidence has demonstrated that diols of some fatty acids such as linoleic acid can be significantly more toxic as compared to their progenitor epoxides [326]. Therefore, it remains unknown whether the EDPs are responsible for the cellular response we observed or if it is attributed to sEH-derived diol metabolites. Nevertheless, EDPs have potential therapeutic value as they possess strong biological effects and are relatively stable chemically [42, 45]. In summary, we show that PPARδ signaling leading to the *de novo* synthesis of ceramide appears to be a crucial step in a DHA-induced cytotoxic cascade in H9c2 cells. Furthermore, our findings demonstrating inhibition of CYP oxidase activity and accumulation of ceramide significantly adds to our understanding of the biological function of epoxy metabolites of DHA. This study provides new insights to understanding the unknown properties of n-3 lipids.

CHAPTER 3

CONCLUDING REMARKS AND FUTURE DIRECTIONS

CONCLUDING REMARKS

The current thesis supports the notion that n-3 PUFAs such as DHA can display cytotoxicity towards immortalized cells. DHA can increase cytotoxic cell death in cancer cells both *in vivo* and *in vitro* via various mechanisms such as increased ROS production and lipid peroxidation [96, 109]. Inconsistencies in the clinical literature regarding the effects of n-3 PUFAs may be attributable to many variables such as age and previous history of CVDs [70]. Another major source of confound and discrepancy in previously published studies regarding n-3 PUFA supplementation has been the baseline nutritional status of the study participants, and in particular, their baseline dietary intake of n-3 and n-6 PUFAs, from fish and other sources which varies by culture [327, 328]. These factors should be kept in mind when interpreting data from experimental and clinical studies. Based on the evidence provided in this thesis it is clear that DHA-mediated signaling is vital to the induction of apoptotic cell death, which can also occur in response to DHA-derived CYP metabolites. There are very few known associations between n-3 PUFAs and ceramide. Thus, our finding that H9c2 cells display DHA-induced accumulation of *de novo* ceramide adds to this expanding area of research, and has implications for future studies utilizing immortalized cells, and potentially for cancer studies as well. DHA induces apoptosis in H9c2 cells through a very specific cell signaling cascade, acting via PPARδ signaling leading to formation of ceramide, and culminating in cell death. We have thus elucidated a specific and novel signaling pathway.

The use of the MS-PPOH to inhibit CYP 450 oxidase activity and subsequent DHA-mediated cytotoxicity, suggests that 19,20- EDP, and not DHA, is the end effector in mediating cell death (see Figure 6). This adds validity to the potential use of EDP as an original target, which can be important for prospective studies, and drug design.

FUTURE DIRECTIONS

n-3 PUFAs can be a powerful adjuvant cancer therapeutic as they typically have either none, or very low recognizable toxicities *in vivo* [99]. Murine data suggests that DHA is extremely well tolerated, as it has been shown to be safe in rats after 90 days even in doses as high as 3290 mg/kg [329], which would translate to ~200 g/d for a 61 kg adult. Clinically, doses as high as 6g/d of n-3 PUFAs are needed in order to modify some markers of CVD such as platelet function, suggesting that doses in some studies such as the OMEGA trial which gave less than 1g/d may be too low, and 2-4 g/d may be more optimal [330]. Side effects are a concern with high doses; however this is not supported by clinical studies. One of the few side effects which has raised some concerns against the clinical administration of high doses of n-3 PUFAs is the possibility of increased bleeding time due to anti-thrombotic properties, along with an additive effect in combination with aspirin [331, 332]. However, it is typically accepted that an intake below 4 g/d does not induce this affect [333]. A wide range of doses have been utilized in humans studies using n-3 PUFAs,

with some higher dosed studies utilizing 7.5g/d, which did not produce any negative influence on lipid levels, platelet function, or immune function [329]. DHA consumption appears to be particular safe, as it is generally accepted that between 4-6g/d (and possibly higher) can be well tolerated [329]. One study demonstrated a dose as high as 9.6 g/d can be well tolerated among patients [334]. Overall, it appears justifiable to recommend the consumption of fish oil for the prevention and treatment of CVDs and cancers. These results suggest that DHA possess very few side effects, and is safe for use even at relatively large doses. As demonstrated in this thesis DHA displays the ability to induce cell death in immortalized cells via PPARδ-mediated signaling, which advocates for future studies to examine this pathway. Furthermore, 19,20-EDP was able to induce apoptosis at a 100-fold lower concentration than DHA supporting its role as a potent lipid mediator. This can be particularly useful for future animal studies. Numerous studies add DHA to animal diets to attempt to deduce its cardioprotective mechanistic actions *in vivo*, but to our knowledge, studies have yet to utilize CYP-derived metabolites such as EDPs in this role. Thus, future research should aim to use 19,20-EDP in feeding studies, which could potentially lead to significant attenuation of cell death within the heart.

Some n-3 PUFA metabolites can act as potent lipid mediators with ~1000 –fold greater potency than DHA and EPA, suggesting that they deserve greater attention when designing experimental as well as clinical studies to look at cell death mechanism and protection of the heart [335]. Hopefully this research

will lead to novel insights into how these FAs either stimulate or protect against cell death, with the goal of applying the knowledge of these cellular signaling mechanism to animal studies.

It was recently demonstrated that 19,20 –EDP administration in combination with a sEH inhibitor via osmotic minipumps lead to the inhibition of growth and metastasis in primary murine tumors [45]. However, the exact molecular mechanisms were not explored. It would be extremely interesting to use a similar animal model in future studies, and to elucidate the role of PPAR-mediated signaling, and if downstream formation of *de novo* ceramide is involved in this protective effect. In addition, we have recently established a protocol to measure EET and DHA-induced alterations in membrane fluidity (discussed in the appendix), which could provide key information regarding signaling pathways leading to apoptotic cell death.

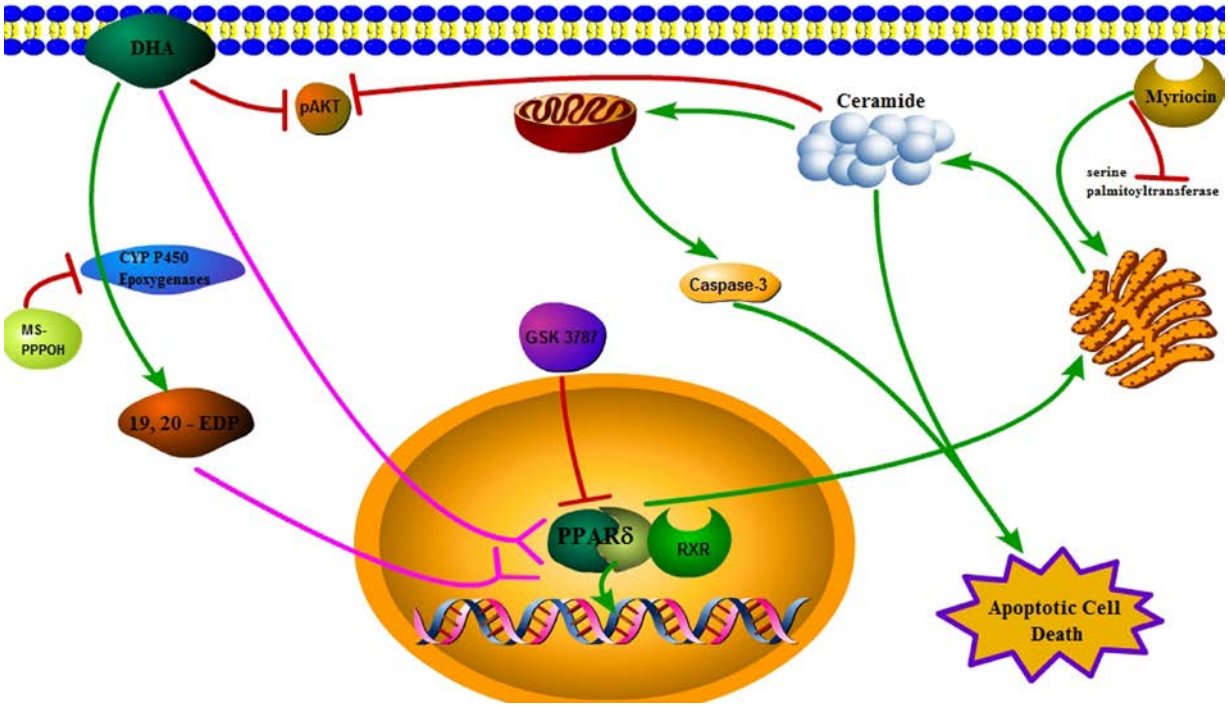

Figure 3.1: Schematic illustration outlining the proposed mechanisms of DHA cytotoxicity in H9c2 cells.

We hypothesize that DHA signaling stimulates the activation of PPARδ signaling. CYP-derived DHA metabolites such as 19,20 –EDP can also stimulate PPARδ signaling. Since MS-PPOH can inhibit both DHA and 19,20-EDP cytotoxic effects, it suggests a vital role for CYP metabolites of DHA. Resulting activation of PPARδ by CYP-derived metabolites of DHA leads to changes in proteasomal activity, phosphorylation of Akt, cellular viability, and caspase-3 activity. Furthermore, it appears that stimulation of PPARδ by 19,20-EDP (or an EDPs mixture) induces significant cell death and formation of ceramide. In summary, excessive activation of PPARδ signaling by 19,20-EDP leads to the *de novo* accumulation of ceramide, and subsequent activation of apoptosis.

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APPENDIX

n-3 AND n-6 PUFA-MEDIATED ALTERATIONS TO MEMBRANE FLUIDITY

A.1 INTRODUCTION

Dietary intake of n-3 PUFAs including DHA, whether through increased intake of fatty fish, or algae-derived capsules, or fish oil, have a ubiquitous ability to become rapidly incorporated within phospholipid membranes causing changes to lipid rafts and cellular signaling [336-338]. Lipid rafts, are regions within the cellular membrane that are rich in sphingolipids and cholesterol [339] [340]. Cellular membranes contain various domains, which are composed of numerous types of lipids and proteins [338], and DHA can be incorporated via esterification within phosphatidylethanolamine and phosphotidylcholine phospholipids [338] in particular. Incorporation can also occur in mitochondrial membranes, leading to biophysical changes to their signaling properties [338, 341].

Changes to membrane fluidity have been implicated in a broad range of disease conditions. As opposed to n-6 PUFAs, n-3 PUFAS, and DHA cell membrane levels in particular, were associated with fast frequency brain activity as measured by electroencephalography suggesting a connection to attention deficit hyperactivity disorder [342]. Alterations in cortical DHA levels were associated with a decrease in membrane fluidity in a mouse model of Parkinson's disease [343]. n-3 PUFAs were shown to affect membrane fluidity in breast cancer cells, leading to caspase-induce apoptosis [93]. Moreover, phospholipids within tumor cells are particularly prone to turnover, thus closer approximating active tissues such as the liver, suggesting that exogenously derived n-3 PUFAs could quickly and efficiently be incorporated within the

cellular membrane leading to wide-spread effects [96, 344]. Phospholipids have the ability to turnover at a faster rate as compared to membrane proteins in the ER microsomal membrane, with both occurring independently [345]. Some synthetic phospholipids such as phosphoethanolamine can itself quickly turnover within the cellular membranes of tumors. It was also shown to specifically induce apoptotic cell death in a tumor cell-specific manner *in vitro*, and increased the overall lifespan of mice *in vivo* [346]. It has been previously been suggested that changes in the turnover rate of phospholipids in the cell membrane can signify tumor growth [347]. As previously measured by EPR, DHA was able cause changes in membrane fluidity in L1210 leukemic cells, which made the uptake of DOX more effective [348].

There is a long-standing notion that a mechanistic function of n-3 PUFA-mediated cardioprotection and possible prevention of coronary artery disease includes changes to membrane fluidity [349, 350]. Although there is some evidence demonstrating DHA-mediated changes to membrane fluidity, the exact molecular mechanism(s) by which this occurs, and the downstream results of this remain unexplored. In addition, the hypothesis that DHA metabolites such as 19,20- EDP can influence the regulation of membrane fluidity is completely novel, and is an exciting concept to explore. In this study we sought to investigate the role of various EETs, as well as DHA, and its CYP-derived metabolites within H9c2 cells and mouse hearts, and to assess their ability to modulate membrane fluidity.

A.2 MATERIALS & METHODS

A.2.1 CELL CULTURE

H9c2 cells were cultured in DMEM and supplemented with 10% FBS, 1% penicillin, and streptomycin at 37° C in an atmosphere of (5% $C0_2$/ 95% N_2). Upon reaching ~80% confluency cells were treated with (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11, 12-EET; 1 μM), 14, 15- EET (1 μM), 20-hydroxyeicosatetraenoic acid (20-HETE) (1 μM), DHA (100 μM), an EDP methyl esters regioisomer mixture (1:1:1:1:1:1; 4,5-, 7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP), and 19,20-EDP (1 μM) for 24 h. All drugs were dissolved in ethanol, and control groups were administered vehicle solution (<5% ethanol, ddH_20).

A.2.2 ANIMALS

C57BL/6 age-matched two month old mice were utilized. sEH null (sEH- KO) and littermate wild-type (WT) mice were treated in accordance with the guidelines of Health Science Laboratory Animal Services, University of Alberta. Mice were administered trans4[43Adamantan1ylureidocyclohexyloxy]benzoic acid (*t*-AUCB, 10mg/L) or vehicle in drinking water for 4 days prior to, and 7 days post-surgery. Mice from all groups were subjected to surgical occlusion of the

proximal left anterior descending (LAD) coronary artery. Control animals were treated similarly without undergoing LAD surgeries (i.e. sham). After 7 days, hearts from all groups were excised and dissected into infarct, peri-infarct, and non-infarcted regions, and flash-frozen. Non-infarcted regions were used to assess changes in membrane fluidity amongst the 6 groups, N= 4 per group.

A.2.3 EPR MEASURMENT OF MEMBRANE FLUIDITY

Changes to membrane fluidity were estimated based on a modified protocol as previously described [351, 352]. After 24 h of treatment, cells were trypsinized, collected, and spun at 500g x 5 min. Cells were then washed with PBS, and spun again at 500g x 5min. Following this, cells were resuspended in a hypotonic buffer (10mM HEPES pH 7.9, 1.5mM $MgCL_2$, 1mM KCL, protease and phosphate inhibitors), and combined with diethylene triamine pentacetic acid (DTPA, 500 μM) dissolved in 500 μM NaOH. Samples were snap frozen in liquid nitrogen, and thawed on wet ice the day of measurement. Once thawed, samples were homogenized on wet ice for 2min, and centrifuged at 2000g x 3min. A protein assay was performed on the supernatants, and all samples were standardized via dilution with the hypotonic buffer to 1μg/μL. Preparation of heart samples for EPR followed the same protocol. Samples were then incubated with the spin proved 5-nitroxyl stearate (5-NS, 1mM) at RT for 30min before EPR analysis.

A.2.4 STATISTICAL ANALYSIS

Values are expressed as means ± SEM. Statistical significance was determined by the use of ANOVA. To determine whether significant differences exist between the groups, a Bonferonni post hoc test was performed. Values were considered significant when $P < 0.05$.

A.3 RESULTS AND DISCUSSION

In these experiments we investigated whether various EETs and n-3 PUFAs can induce changes to membrane fluidity *in vitro* and *in vivo*. We found that 11, 12- EET, 14,15-EET, 20-HETE, 19,20- EDP, and DHA induced significant increases in membrane fluidity, as assessed by changes to the spin probe 5-NS using EPR analysis. This suggests that n-3 PUFAs such as DHA induce changes in membrane fluidity in immortalized cells after 24h of treatment. Although both DHA and EDPs significantly increased membrane fluidity, the effects of DHA were not necessarily due to its conversion to EDPs, as DHA can also be kept as a FFA without further metabolism [73], suggesting that both DHA and 19,20-EDP may function to alter membrane fluidity. Interestingly, the change in fluidity in response to 19,20-EDP was not as dramatic as compared to DHA, but 19,20-EDP was able to induce a significant increase in membrane fluidity at a 100-fold lower concentration (Figure 6A, C-F). This suggests that 19,20- EDP may mediate its previously discussed cytotoxic effects via alterations in membrane fluidity.

t-AUCB given to control mice significantly increased membrane fluidity, as compared to control mice not administered t-AUCB. This suggests that pharmacological inhibition of sEH alone can influence membrane fluidity responses. In addition, t-AUCB given to sEH KO mice induced a significant increase in membrane fluidity as compared to control mice that were provided t-AUCB, suggesting an additive effect between genetic and pharmacological

inhibition/ knockout of sEH. This has important implication for CVD as sEH inhibition is a bona-fide target for the prevention of I/R injury [353, 354], thus suggesting that alterations in membrane fluidity may play a key role in the protection afforded by sEH KO. Another important point to consider is that although we did not directly assess the amount of 19,20-EDP within the heart tissues, we hypothesize that significant alterations to its levels probably occurred. This is because sEH further metabolizes EDPs into inactive diols [39]. Therefore, inhibition of sEH in our experiments by the use of t-AUCB would lead to significantly increased levels of 19,20-EDP. This is preliminary data and results are not definitive, thus further exploration in other cells lines, such as primary rat neonatals is needed in order to understand the role that n-3 PUFAs and their metabolites have leading to biophysical alterations to fluidity. This research can potentially be used in future studies in combination with DHA or its metabolites such as 19,20- EDP to measure changes to membrane fluidity in various cell lines and cardiac models, *in vitro* and *in vivo*, respectively.

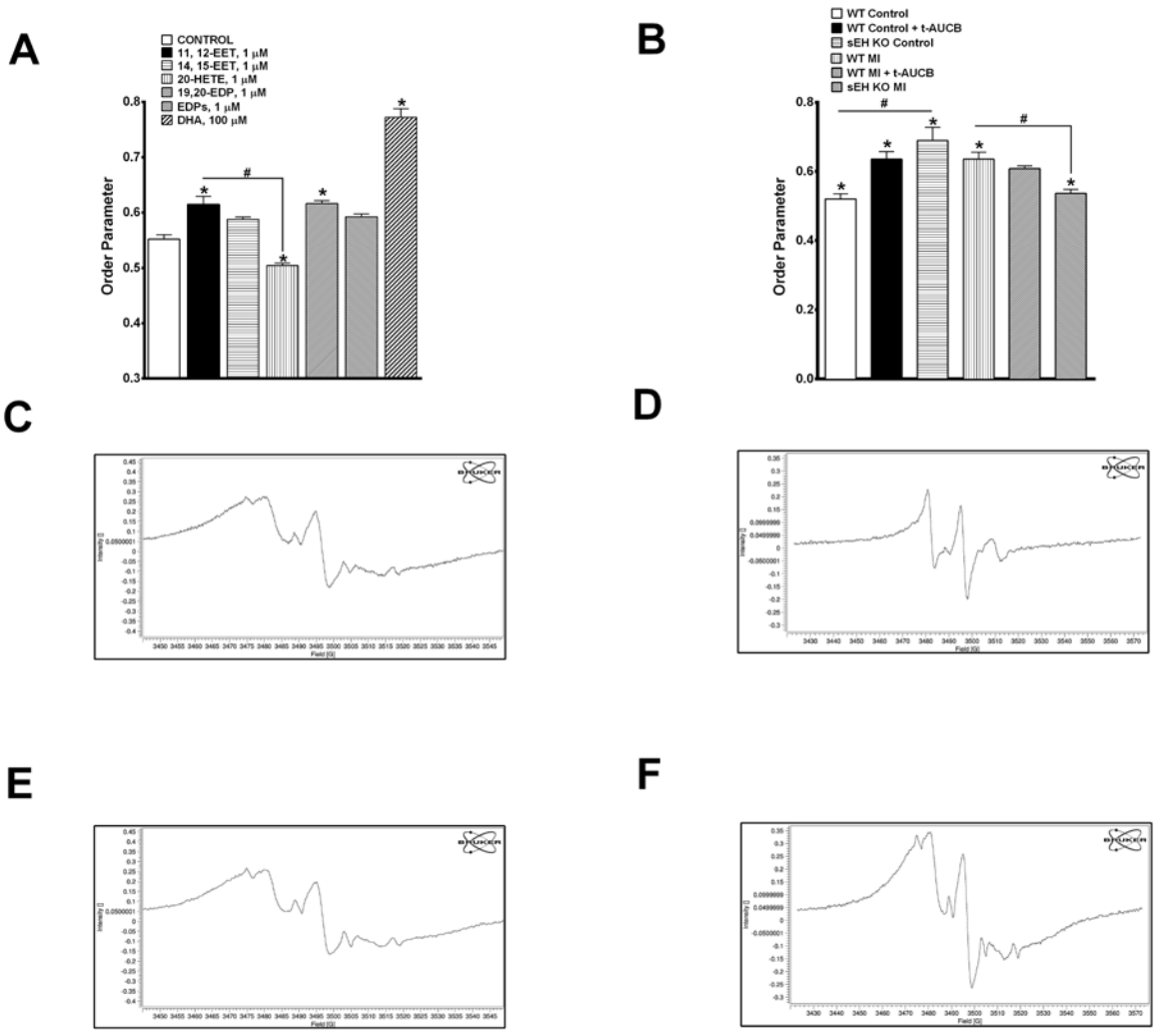

Figure A.1: EET and DHA-induced changes in membrane fluidity. (A) H9c2 cells were treated with either 11, 12-EET 1 μM, 14, 15-EET 1 μM, 20-HETE 1 μM, DHA 100 μM, EDPs mixture 1 μM, or 19,20 – EDP 1 μM , and assessed at 24h for changes in membrane fluidity. Values are expressed as mean ± SEM; N=3; *, $p<0.05$ 11, 12- EET, or 14-15- EET, or 19,20-EDP, or DHA vs. control, #, $p<0.05$ 11, 12- EET vs. 20-HETE . (B) Post-MI non-infarcted tissues were excised in mice administed t-AUCB within their drinking water. Values are expressed as mean ± SEM; N=4; *, $p<0.05$ WT control + t-

AUCB, or sEH KO control + t-AUCB, or WT+MI, or sEH KO+MI, vs. control, #, $p<0.05$ WT control vs. sEH KO control or WT+MI vs. sEH KO+MI. (C-F) Representative raw image traces for H9c2 cell treatment groups; control, EDPs mixture, 19,20-EDP, and DHA, respectively.