The protective effects of stearidonic acid on adipogenesis and neurotoxicity

by

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Abstract

The ω -3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), in fish oil have been reported to have protective effects on metabolic syndrome-related diseases, such as obesity and alzheimer's disease (AD). Stearidonic acid (SDA) is a plant-based ω-3 PUFA that serves as the metabolic precursor of EPA. SDA has been shown to have similar metabolic effects to DHA and EPA. This dissertation was aimed to investigate whether SDA has similar effects to DHA and EPA on obesity and AD.

Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from preadipocytes. The ω-3 PUFAs found in fish oil have been shown to have anti-obesity effects through inhibition of adipocyte differentiation. In the 3T3-L1 cell model, we demonstrated that SDA treatment led to significantly greater EPA enrichment compared to ALA-treated cells. In addition, SDA treatment, similar to EPA and DHA, inhibited fat storage in 3T3-L1 cells indicated by decreased accumulation of lipid droplets and reduced triglyceride (TG) content. Further, we demonstrated that this anti-adipogenic effect by SDA may rely on its down-regulation of mRNA levels of the adipogenic transcription factor, SREBP-1c, and the lipid accumulation genes, AP2, FAS, SCD-1, LPL, GLUT4, and PEPCK. In summary, SDA is able to suppress the adipocyte differentiation and lipid accumulation by affecting the expression of associated genes. Our findings warrant further study to develop SDA as a natural and effective agent in the prevention or treatment of obesity.

AD involves an amyloid-beta (Aβ)-induced cascade of an increase in apoptosis, oxidative stress, and inflammation. DHA and EPA have been shown to protect against Aβ-induced neurotoxicity through modulation of this cascade. In the H19-7 hippocampal cell model, we demonstrated that SDA treatment led to significantly greater EPA enrichment compared to ALA-treated cells. Moreover, SDA significantly decreased neuronal death by inhibition of caspase activation and regulation of apoptotic Bcl-2 family gene expression. Total anti-oxidant capacity was significantly increased in SDA-treated cells through increased catalase activity and up-regulation of anti-oxidative gene expression, such as GPx, GSR, and SOD. SDA significantly decreased Aβ and LPS-induced expression of pro-inflammatory mediators, IL-6, TNFα, COX-2, MCP-1 and TLR4. SDA exerted neuroprotective effects through attenuation of Aβ-induced JNK and p38 phosphorylation, and enhancement of ERK phosphorylation depressed by Aβ. Importantly, the efficacy of neuroprotective effects by SDA is more significant than that of ALA, and comparable to that of EPA and DHA. In summary, SDA protects against Aβ-induced neurotoxicity in hippocampal neurons. Our findings warrant further study to develop SDA as a natural and effective agent in the prevention or treatment of AD.

It is known that ω-3 PUFAs exert their biologic activities via formation of eicosanoids by competing with ω-6 PUFAs. Generally, ω-3 derived eicosanoids are less potent than analogous ω-6 derived eicosanoids. However, to date, only a few studies have investigated the overall effects on eicosanoid production by ω-3 fatty acids and no study has been conducted on SDA treatment. The present study conducted a lipidomics study to characterize the changes in eicosanoid profile in H19-7 rat hippocampal cells upon treatment of different ω-3 fatty acids. Our results showed that 1) ω-3 fatty acids affect the production of eicosanoids by LOX, COX, CYP, and non-enzymatic autoxidation pathways; 2) ω-3 fatty acids are natural anti-inflammatory compounds that

can convert to a lot of anti-inflammatory eicosanoids under normal conditions; 3) ω-3 fatty acids, especially EPA can also increase the production of many pro-inflammatory metabolites; 4) treatment of DHA can increase the ROS production. In general, the effects on eicosanoid production are consistent among all four ω-3 fatty acids and the efficacy of anti-inflammatory potent is DHA&EPA > SDA >> ALA. Particularly, the changing pattern of eicosanoids affected by SDA treatment is most close to the pattern by EPA, which confirmed the proposal that SDA is the surrogate for EPA. Importantly, compared to EPA, SDA is much less potent in increasing the production of anti-inflammatory metabolites, but also much less potent in increasing the production of pro-inflammatory metabolites, suggesting that SDA may have advantages over EPA. Moreover, data suggest that SDA has its unique roles in biosynthesis of eicosanoids, such as decreased PGD1 level, suggesting that SDA may have its own specific biological functions different from DHA and EPA. Finally, this lipidomics study of ω-3 fatty acids is valuable and provides lots of inspirations for future studies, especially in the area of chronic inflammatory diseases, the area of cardiovascular system where ω-3 fatty acids act as vasodilator, and the area of host immune defense.

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List of Abbreviations

AD	alzheimer's disease
ALA	alpha-linolenic acid
APP	amyloid precursor protein
AP2	adipocyte fatty acid binding protein
Aβ	amyloid-beta
BACE1	β-Site APP-cleaving enzyme 1
BAT	brown adipose tissue
BBB	blood-brain barrier
Bcl-2	B-cell lymphoma-2
BMI	body mass index
CREB	cAMP response element binding protein
COX	cyclooxygenase
CRP	c-reactive protein
CVD	cardiovascular disease
CYP	cytochrome P450
C/EBP	CCAAT-enhancer-binding protein
DEX	dexamethasone
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid

EPA	eicosapentaenoic acid
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
GLUT	glucose transporter
GPx	glutathione peroxidase
GSR	glutathione reductase
HDL	high density lipoprotein
IBMX	3-isobutyl-1-methylxanthine
IDE	insulin-degrading enzyme
IGF-1	insulin-like growth factor-1
IL	interleukin
JNK	jun N-terminal kinase
LC/MS	liquid chromatography/mass spectrometry
LOX	lipoxygenase
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
PEPCK	phosphoenolpyruvate carboxykinase
PPAR	peroxisome proliferator activated receptor)
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SCD	stearoyl-CoA desaturase

SDA	stearidonic acid
SOD	superoxide dismutase
SREBP	sterol regulatory element-binding protein
TG	triglyceride
TLR	toll-like receptor
TNF	tumor necrosis factor
WAT	white adipose tissue

Chapter 1 Introduction

Omega-3 PUFAs are considered essential fatty acids as they cannot be synthesized de novo. They are vital for normal metabolism and need to be supplied from diets. Alpha-linolenic fatty acid (ALA), which can be found in plants, is the precursor of other ω-3 PUFAs, SDA, EPA, and DHA. SDA is also plant-sourced, while DHA and EPA are mainly found in fish, such as salmon and tuna, and other seafood including algae and krill, known as marine-sourced fatty acids. Adequate fish intake in our diet provides an optimal supply of ω-3 PUFAs for metabolism since in vivo conversion of ALA to DHA and EPA might be limited. Deficiency in ω-3 PUFAs is characterized by fatigue, poor memory, dry skin, heart problems, and depression. Omega-3 PUFAs play a crucial role in normal growth and development, especially in brain functions. In fact, ω-3 PUFAs are highly concentrated in the brain and appear to be important for cognitive and memory function. Infants are at risk for developing vision and nerve problems when limited access to ω-3 PUFAs from their maternal sides (1, 2). Moreover, it is also well-established that ω-3 PUFAs may reduce the risk of heart disease (3). A consumption of two to three servings per week of fish rich in ω-3 PUFAs is suggested since it should provide about 500 mg/day of DHA and EPA for primary prevention of cardiovascular disease (CVD) in adult general population (4). For secondary prevention, patients with documented CHD should consume approximately 1 g EPA + DHA/day from oily fish or fish oil capsules (5). In addition, as the precursor of anti-inflammatory molecules, ω-3 PUFAs were shown to reduce inflammation and may help lower risk of inflammatory diseases, such as arthritis (6). In contrast, ω-6 fatty acids tend to promote inflammation. Therefore, a

balanced ω-6 to ω-3 fatty acid ratio in the diet is important for health. However, the typical American diets usually have a ratio of 8-17:1 for ω-6 to ω-3, which is considered to be way too high on ω-6 side and may be associated with certain diseases, such as metabolic syndrome, CVD, and inflammation (4). Diet with a ratio of 4:1 for ω-6 to ω-3, which is associated with a 70% decrease in total mortality of heart disease (7) is recommended. This dissertation will focus on the effects on obesity and AD by ω-3 fatty acids.

Obesity is increasing dramatically and becomes the most significant public health problem. Low-grade inflammation has been identified as a key factor in the development of obesity and obesity-related diseases (8). Enhanced inflammatory activity of adipose tissue in obesity attributes to macrophage infiltration and increased levels of circulating adipokines secreted by adipocytes, such as IL-6, TNFα, and MCP-1 (9). Long-chain ω-3 PUFAs play an important regulatory role in immune responses and inflammation. In general, long-chain ω-3 PUFAs, especially DHA and EPA, are incorporated into cell membrane phospholipids and converted to eicosanoids, resolvins, protectins and maresins, which are potently anti-inflammatory (10). Moreover, ω-3 PUFAs can compete with ω-6 PUFAs, which form a different class of eicosanoids with pro-inflammatory properties, thus can counteract the effects of the ω-6 PUFAs-derived eicosanoids (11). Previous studies suggested that plasma ω-3 PUFAs are negatively associated with obesity, whereas ω-6 PUFAs are positively associated with obesity (12, 13). Significantly increased ω-6 to ω-3 PUFAs ratio was reported in the liver of patients with non-alcoholic fatty liver disease (14). Consumption of ω-3 PUFAs are associated with reduced body weight and fat mass. The inclusion of either lean or fatty fish, or fish oil as part of an energy-restricted diet could result in approximately 1 kg more weight loss after 4 weeks, than did a similar diet without seafood or supplement of marine origin, in young and overweight men (15). In addition, consumption of ω-3 PUFAs are associated with

an improvement of lipid profile and other metabolic syndrome features. Fish or fish oils were reported to decrease plasma triacylglycerol and increase HDL cholesterol in insulin-resistant obese men (16) and in overweight hypertensive subjects (17). The insulin sensitivity of patients with non-insulin-dependent diabetes has been found to be improved by dietary supplementation of ω-3 PUFAs (18). In healthy older adults, high consumption of ω-3 PUFAs could increase insulin sensitivity and reduce inflammatory markers, CRP and IL-6 (19).

AD is now recognized as type 3 diabetes since an association between diabetes and increased risk of AD has been demonstrated (20). Present evidence suggests that onset of cognitive impairment in AD could have major underlying processes that cause neuronal death, including defective antioxidant defense mechanisms, inflammatory processes, and reduced mitochondrial energy production. Long-chain ω-3 PUFAs have been found to exert beneficial effects on cognitive function in patients with early onset mild cognitive impairment (21). While, intake of saturated fatty acids have been found to induce cognitive decline (22). Minimizing consumption of saturated fats and trans-fats has been listed in the guidelines for the prevention of AD (23). Lower contents of ω-3 PUFAs have been measured in the plasma and brain of patients with AD compared with normal healthy individuals (24-28). Normal physiological changes to the brain with aging include depletion of major long-chain ω -3 PUFAs, which is accelerated in neurodegenerative conditions such as AD (29). Epidemiological observations suggest that high ω-3 PUFA intake from fatty fish or fish oil is associated with decreased risk of mild cognitive impairment (30). In animal studies, the beneficial effects of ω-3 PUFA were observed particularly on Aβ deposition, tau pathology, neuron loss, and cognitive deficits. Ikemoto and colleges found that the altered learning behavior associated with a long-term ω-3 PUFA deficiency could be reversed by supplementing the diet with DHA-enriched oil (31). Wu and colleges demonstrated

that the EPA-enriched phospholipids administration prevented the development of learning and memory impairments in SAMP8 mice, and indicated that this neuroprotective effect may rely on the anti-oxidative activity and inhibition of mitochondria-dependent apoptotic pathway of EPA (32). Although the molecular basis for such protective effects of ω-3 PUFAs remains unclear, conclusion from the investigations in animal models and cell culture usually described to rely on 5 distinct and interconnected molecular mechanisms (33): (i) regulation of gene expression for various proteins involved in Aβ production and tau pathology (34), (ii) reduction of oxidative damage (32), (iii) block of inflammatory cascade (35), and (iv) inhibition of cell apoptosis pathways (36), as well as (v) membrane remodeling (37).

Cold water fish and fish oils are the most direct source of DHA and EPA. However, oily fish consumption in Western countries including the United States remains low (38). Due to concerns regarding the fish palatability (39), global stagnation in the fishery industry (40), and possible methyl mercury contamination in some fish species (41), there is a need and desire to identify and develop alternative sources of EPA and DHA that have similar biological properties. ALA is the main ω-3 fatty acid available in vegetable oils. Dietary ALA goes through the reactions of desaturation and elongation to convert to EPA and DHA. Due to the rate-limiting enzyme-Δ6 desaturase, the conversion from ingested ALA to EPA and DHA is so poor that currently there is insufficient evidence that ALA has health benefits similar to EPA and DHA (42). SDA represents the Δ6 desaturation product of ALA, and thus bypasses the rate-limiting step in the conversion of dietary ALA to EPA and DHA. Due to its relatively efficient conversion following consumption, SDA has been targeted as a potential biologically active surrogate for EPA (43).

SDA is rich in the seeds and leaves of several plants, such as echium plantagineum, borage (*boragoofficinalis*), evening primrose (*oenotherabiennis*), and blackcurrant (*ribesnigrum*).

Recently, genetic-modified SDA-enriched soybean oil has become the most common source of SDA with improved SDA content of 20-30% (44). Studies have shown that SDA was able to improve lipid profile (45, 46), decrease leukotriene generation (47), inhibit inflammation (48), increase the cardiovascular risk marker omega-3 index (49), attenuate hepatic steatosis (45), reduce atherosclerosis (46) and slow cancer growth (50). However, the effects of SDA on obesity and AD are unknown. Therefore, in the present study, we hypothesized that SDA, as a precursor of DHA and EPA, on one hand, will suppress adipocyte differentiation and reduce fat deposition in 3T3-L1 cells through alterations in the expression of genes connected with lipid metabolism (Chapter 3); on the other hand, will suppress apoptosis, oxidative injury, and inflammation induced by Aβ in H19-7 hippocampal cells through the same mechanisms by which DHA and EPA protect against neurotoxicity (Chapter 4).

Supplementation of long-chain ω-3 PUFA into the human diet contributes to anti-inflammatory properties and benefits for certain chronic inflammatory conditions. It is known that ω-3 PUFAs exert their biologic activities mainly via formation of eicosanoids by competing with ω-6 PUFAs. Generally, ω-3 derived eicosanoids are less potent than analogous ω-6 derived eicosanoids. However, to date, only a few studies have investigated the overall effects on eicosanoid production by ω-3 fatty acids, mainly DHA and EPA; no study has been conducted on SDA treatment. Therefore, in order to better understand how ω-3 fatty acids work after incorporation into the cell membrane, the present study employed lipidomics, a large-scale study of pathways and networks of cellular lipids in biological systems, to characterize the changes in eicosanoid profile in H19-7 rat hippocampal cells upon treatment of different ω-3 fatty acids (Chapter 5).

Chapter 2 Review of literature

2.1 Obesity

2.1.1 Epidemiology, causes, consequences, and strategy

Obesity is one of the biggest drivers of chronic diseases in the United States. The World Health Organization and the National Institutes of health have defined overweight as having a body mass index (BMI) between 25.0 and 29.9 kg/m^2; and obesity as having a BMI greater than 30.0 kg/m^2 (51). The last few decades have seen a dramatic rise in the prevalence of overweight and obesity in the United States across all ages. Between 2011 and 2014, the prevalence of obesity was over 36% in adults and 17% in youth in the United States (52). Obesity is now recognized as one of the most important public health concerns of our time and places a considerable burden on healthcare systems. Currently, the estimated costs range from $147 billion to nearly $210 billion per year (53).

Obesity results from a complex interaction between the environment, genetics, and individual behavior. People and families may make decisions based on their environment or community. It has been shown that the temporal trends in the increase of the quantity and energy density of foods parallel the increasing prevalence of obesity in the U.S. population (54). Car ownership and television viewing, proxy measures of physical inactivity, were found to closely parallel the rising trends in obesity in England (55). A person's risk of becoming obese increased by 57%, 40%, and 37% if a friend, a sibling, and a spouse became obese, respectively (56). Therefore, it is important to create environments in these locations that make it easier to engage in

physical activity and eat a healthy diet. In addition to environmental factors, there is genetic predisposition to obesity. Genes give the body instructions for responding to changes in its environment. Studies have identified variants in several genes that may contribute to obesity by increasing hunger and food intake. Monogenic obesity described as rare and severe early-onset obesity within a family, which caused by a specific variant of a single gene, such as leptin, leptin receptor, melanocortin-4 receptor, and pro-opiomelanocortin (57). However, most obesity probably results from complex interactions among multiple genes and environmental factors that remain poorly understood (58). Obesity develops when there is a positive imbalance between energy intake and energy expenditure. Individual behaviors can contribute to excess caloric intake and inadequate amounts of physical activity. Behaviors can include dietary patterns, physical activity, medication use, and other exposures. Significant correlations between women's BMI and age, race, dietary patterns, and smoking were found in a previous study (59). Increased snacking and eating away from home, larger portion sizes, greater exposure to food advertising, and limited access to physical activity opportunities were all found to attribute to the current high rates of obesity (60-62). Inadequate sleep, maternal pre-pregnancy weight status, maternal smoking during pregnancy, and stress may also affect energy balance or obesity risk (63-65).

Obesity is a serious concern associated with poorer mental health outcomes, reduced quality of life, and the leading causes of death in the U.S. and worldwide. It was reported that the risk of death among patients who had never smoked would increase by 20% to 40% in overweight patients and by 2- to 3-fold in obese compared with normal-weight patients in a prospective cohort of more than 500,000 U.S. men and women after 10 years of follow-up (66). Obesity is also associated with increased risk for numerous chronic diseases including the following: diabetes, hypertension, dyslipidemia, coronary heart disease, stroke, gallbladder disease, osteoarthritis,

sleep apnea and breathing problems, mental illness, liver and kidney disease, and some cancers (67).

There is no single or simple solution to the obesity epidemic. It's a complex problem and there has to be a multifaceted approach. These include strategies such as modifying the design of buildings to encourage the use of stairs, establishing more walkable neighborhoods, increasing the range of healthy foods in schools and work cafeterias, and improving food labeling to help consumers to make informed choices (68). Nutrition education with a strong emphasis on reducing the consumption of fat while increasing that of fruit and vegetables has been demonstrated a useful approach to prevent obesity (69). A healthy diet pattern which emphasizes eating whole grains, fruits, vegetables, lean protein, low-fat and fat-free dairy products and drinking water is recommended with the Dietary Guidelines for Americans (70). The Physical Activity Guidelines for Americans recommends adults do at least 150 minutes of moderate intensity activity or 75 minutes of vigorous intensity activity, or a combination of both, along with 2 days of strength training per week (71).

2.1.2 Obesity and adipocyte differentiation

Obesity is a condition characterized by excess body fat mainly in adipose tissue that results from positive energy balance and is the most common metabolic disorder in the world. In mammals, two types of adipose tissue exist: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is responsible for lipid uptake, synthesis, and storage in the form of TG (72). When fasting, TG stored in WAT will be mobilized and broken down into fatty acids and glycerol, serving as an energy source. BAT functions to maintain body temperature by efficiently dissipating stored energy as heat. BAT is abundant in newborns, protecting against exposure to cold temperature, and largely disappears during childhood with little interspersed WAT throughout adulthood (73).

Adipose tissue is mainly composed of adipocytes. White adipocytes usually contain one major lipid droplet that fills up the majority of the cytoplasm and relatively few mitochondria. In contrast, several small lipid droplets and a large number of mitochondria exist in brown fat cells (74). In obesity, TG tends to be accumulated in adipose tissue, disrupting the normal function of adipocytes, leading to higher release of free fatty acids into the circulation, and ectopically accumulation of TG in liver and muscle, and thus triggers insulin resistance and metabolic disorders in the whole body (75).

Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells, which allows adipose tissue also acts as an active endocrine organ that produces a variety of humoral factors, known as adipocytokines, which in turn exert numerous metabolic and vascular effects. Under the obese condition, adipose tissue produces and releases a variety of adipocytokines with pro- or anti-inflammatory properties, including leptin, adiponectin, resistin, visfatin, as well as cytokines and chemokines, such as TNF-α, IL-6, IL-1β, monocyte chemoattractant protein 1 (MCP-1), plasminogen activator inhibitor-1, and others (76). Changes in the release of these adipokines is further associated with the complex network of various biological responses, including inflammation, immunity, endocrine, and metabolism (75).

Obesity is defined as an excessive accumulation of fat mass in adipose tissue to the extent that health may be adversely affected, which can occur through an increase of adipocyte volume (hypertrophy) and number (hyperplasia) (77). This process is known as adipocyte differentiation. A thorough understanding of the differentiation process would allow for manipulation of adipocyte cell number and control of certain diseases, such as obesity. Adipocyte differentiation, or adipogenesis is a complex and programmed process in which new adipocytes are derived from

preadipocyte precursors (78). Briefly, adipocyte differentiation is divided into four steps, including initial growth arrest, mitotic clonal expansion, early differentiation, and terminal differentiation–development of mature adipocyte phenotype (74). Preadipocytes retain the capability to differentiate into adipocytes in vitro (79). The 3T3-L1 cell line is one of the most well-characterized and reliable models for studying the conversion of preadipocytes into adipocytes. Confluent 3T3-L1 preadipocytes can be differentiated synchronously by a defined adipogenic cocktail, which includes insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX) (80). After the first 48-72 h, insulin alone is required to continue the differentiation program. Insulin is known to act through the insulin-like growth factor 1 (IGF-1) receptor. DEX, a synthetic glucocorticoid agonist, is used to stimulate the glucocorticoid receptor pathway. IBMX, a cAMP-phosphodiesterase inhibitor, is used to stimulate the cAMP-dependent protein kinase pathway. Upon cocktail induction, 3T3-L1 preadipocytes can differentiate into mature adipocyte cells, expressing specific adipocyte genes and accumulating triacylglycerol lipid droplets (81).

Adipogenesis is a complex process in which a cascade of transcription factors and cell-cycle proteins modulating hundreds of genes responsible for adipocyte development. In response to adipogenic stimuli, C/EBPβ and C/EBPδ are transiently activated within 24 h and directly induce expression of PPARγ and C/EBPα, the key transcriptional regulator of adipocyte differentiation (82). PPARγ and C/EBPα initiate positive feedback to induce their own expression and also activate a large number of downstream target genes whose expression determines the adipocyte. Ectopic expression of C/EBPα in various fibroblast cell lines promotes adipogenesis only in the presence of PPARγ (83). In contrast, PPARγ ectopic expression can induce adipogenesis in mouse embryonic fibroblasts lacking C/EBPα, indicating that PPARγ, but not C/EBPα, is sufficient to induce adipogenesis (84). C/EBPα and PPARγ regulate each other's

expression and form a positive feedback loop that plays pivotal roles at the later stage of adipocyte differentiation by inducing and maintaining the expression of multiple adipocyte-specific genes, including adipocyte fatty acid binding protein (AP2), CD36, fatty acid transport protein 1 (FATP-1), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), lipoprotein lipase (LPL), glucose transporter 4 (GLUT4), and phosphoenolpyruvate carboxykinase (PEPCK) (74, 85). Activation of PPARγ has been shown to promote adipose expansion and TG accumulation in adipose tissue (86). In addition to PPARγ and C/EBP family members, a number of other transcription factors are likely to play a role in regulating adipogenesis. Kruppel-like factor (KLF) was presumably to function through the differential recruitment of co-repressors and co-activators to the PPARγ-2 promoter (87). Sterol regulatory element binding transcription factor 1 (SREBP1c) was identified as a pro-adipogenic transcription factor that induces PPARγ expression (88). SREBP1c also mediates the insulin-triggered lipid biosynthesis in adipocytes by increasing the gene expression of the main lipogenic genes, such as FAS and acetyl-CoA carboxylase (89). The cAMP/PKA/CREB signaling pathway affects adipocyte differentiation through regulating C/EBPβ (90). Silencing of CREB expression blocks adipogenesis (91). Other transcription factors that promote adipogenesis include endothelial PAS domain protein 1 (92), the signal transducer and activator of transcription-5a (93), the circadian regulator brain and muscle ARNT-like Protein 1 (94), and paternally expressed gene 1 (95).

2.2 Alzheimer's disease

2.2.1 Epidemiology, causes, pathology, and strategy

AD is one of the most common neurodegenerative disease and accounts for more than 80% of dementia cases worldwide in elderly people. AD is a progressive disease beginning with mild

memory loss possibly leading to loss of mental, behavioral, functional decline and ability to learn (96). According to 2016 Alzheimer's disease facts and figures, an estimated 5.4 million Americans have AD; by 2050, the number of people living with AD in the United States is projected to grow to 13.8 million. Currently, one new case of AD in the country develops every 66 seconds; by 2050, one new case of AD is expected to develop every 33 seconds, resulting in nearly 1 million new cases per year (97). In 2013, AD ranked the sixth leading cause of death in the United States and the fifth leading cause of death in Americans age ≥ 65 years. However, between 2000 and 2013, deaths resulting from stroke, heart disease, and prostate cancer decreased 23%, 14%, and 11%, respectively, whereas deaths from Alzheimer's disease increased 71%. In 2016, an estimated 700,000 Americans age ≥ 65 years will die with Alzheimer's disease, and many of them will die because of the complications caused by Alzheimer's disease (97). AD imposes a severe burden upon patients and their caregivers. Family caregivers of AD patients face extreme hardship and distress that represents a major but often hidden burden on healthcare systems (98). In 2015, more than 15 million family members and other unpaid caregivers provided an estimated 18.1 billion hours of care to people with dementia including AD, a contribution valued at more than $221 billion; in 2016, total payments for health care, long-term care and hospice services for people age ≥ 65 years with dementia are estimated to be $236 billion (97).

AD is caused by a combination of genetic, lifestyle, and environmental factors that affect the brain over time. Aging is the greatest known risk factor for AD. The risk for dementia increases greatly after age 65 and doubles every decade after age 60 (99). People with rare genetic changes linked to early-onset AD begin experiencing symptoms as early as their 30s. Early-onset familial AD is usually caused by autosomal dominant mutations in the genes for amyloid precursor protein (APP), presenilin 1 and presenilin 2, accounting for approximately 2–5% of all AD cases (100).

Most genetic mechanisms of AD among families remain largely unexplained; however, the strongest risk gene found so far is apolipoprotein E4 (apoE4). In fact, the apoE4 allele is the only proven genetic factor so far identified in the development of AD (101). Nowadays, more attention has been focused on the vascular risk factors or lifestyle factors, such as smoking, obesity, high total cholesterol levels, hypertension, diabetes mellitus, and asymptomatic cerebral infarction, which are associated with a higher risk of dementia including AD (102). Many analytical studies have found a significantly increased risk of AD associated with cigarette smoking, especially in apoE4 allele non-carriers (103). Middle-aged heavy drinkers, especially apoE4 allele carriers, were found to have a more than 3-fold higher risk of dementia and AD later in their lives (104). Higher BMI or obesity at around the age of 50 years means an increased risk of dementia 20-25 years later (105). Several studies have shown protective effects of antihypertensive drugs (106) and anti-hypercholesterolemia statin (107) against the development of AD. Studies have shown that decreased risk of AD are associated with higher intake of antioxidants, such as vitamins E and C (108) and the Mediterranean diet (109). It was also reported that a diet rich in saturated fats and cholesterol increased the risk of AD (110); whereas, PUFAs and fish may be protective against dementia (27). Other risk factors for AD are diabetes, CVD, physical activity, and social networks.

The neuropathological hallmarks of AD include amyloid plaque, neurofibrillary tangles, glial responses, and neuronal and synaptic loss. The senile plaque consists of a central core of Aβ, a 4-kD peptide, surrounded by abnormally configured neuronal processes or neurites. Activated astrocytes and microglial cells are commonly associated to amyloid plaques, indicating that Aβ is a major trigger of glial response in brain (111). The neurofibrillary tangle consists of abnormal accumulations of abnormally phosphorylated tau in certain neurons. Neuronal loss and synapse

loss largely parallel tangle formation, although whether tangles are causative of neuronal loss or synaptic loss remains uncertain (112).

To prevent AD, vascular, nutritional, and lifestyle factors should be modified. People are encouraged to take control of hypertension, obesity, increased glucose levels, and diabetes in middle age; to avoid very low blood pressure and maintain sufficient cerebral perfusion; to ensure an extensive social network (102). To treat AD and dementia, cognitive training, cholinesterase inhibitors (donepezil), and N-methyl D-aspartate receptor antagonists (memantine) are all widely used.

2.2.2 Aβ-induced neurotoxicity

In AD, the dysregulation of the Aβ level leads to the appearance of senile plaques, which contain Aβ depositions. Recent evidence suggests that Aβ deposits have a causal role in its pathogenesis, but the underlying mechanisms remain uncertain (113). Amyloid precursor protein (APP) is expressed at high levels in the brain and metabolized in a rapid and highly complex fashion (114). The APP is cleaved by two pathways. In the nonamyloidogenic pathway, the full-length APP is cleaved by α- and γ-secretases. In the amyloidogenic pathway, β-Site APP-cleaving enzyme 1 (BACE1) cleaves APP to produce the C99 fragment and soluble APPβ, and then the C99 is cleaved by γ-secretase to produce neurotoxic forms of Aβ (115). Moreover, presenilin 1 and 2 regulate the proteolytic function of γ-secretase, and mutations in these proteins can increase the ratio of Aβ in early-onset forms of AD (116).

The imbalance of the Aβ level in AD is not only due to its increased production, but also a decreased in its clearance in the brain. There are different pathways such as the activation of degrading enzymes, receptor-mediated cellular and vascular clearance and other mechanisms by

which Aβ is cleared in the brain (117). Studies have shown that conditional knockout of low-density lipoprotein receptor-related protein 1 (LRP1) in mouse forebrain neurons increased brain Aβ levels and exacerbated amyloid plaque deposition selectively in the cortex (118). Ablation of P-glycoprotein, an Aβ efflux pump at the blood-brain barrier (BBB), has been shown to increase Aβ deposition in the brain of an AD mouse model (119). The activity of neprilysin, an Aβ degrading peptide, has been found to reduce in the cortex and hippocampus of AD patients (120). Genetic studies have shown that gene variations of insulin-degrading enzyme (IDE), which also degrades Aβ, are associated with the clinical symptoms of AD (121).

Aβ aggregation involves a transfer of very short oligomers to diffusible ligands and finally protofibrils, which are able to induced neurotoxicity. In particular, Aβ protofibrils may be part of a mechanism controlling synaptic activity, acting as a positive regulator presynaptically and a negative regulator postsynaptically (122). Aβ deposits depress excitatory transmission at the synaptic level, triggering aberrant patterns of neuronal circuit activity and epileptiform discharges at the network level.

On the other hand, Aβ has been shown to interact with a lot of membrane receptors and thereby affect intracellular signaling. By signaling modulation, Aβ is able to provoke nitric oxide formation and an influx of calcium ions which might eventually lead to the formation of peroxynitrite radicals and cell death (123). It has been shown that Aβ increases the expression and activity of ryanodine receptor 3 and subsequently disrupt intracellular Ca^{2+} levels (124). LRP1 has been shown to be involved in Aβ deposition through sequestration and removal of its soluble forms (125). AD progression disrupts the expression of sorting protein-related receptor, which is expressed in neurons and controls APP trafficking (126). Aβ can also bind to CD36, which involved in innate immune, and thereby activate reactive oxygen species (ROS) production,

vasoconstriction, and provoke neuronal damage (127). Moreover, Aβ is an agonist for N-formyl peptide receptor like-1, which activation is associated with neuronal inflammatory response in AD (128). Furthermore, Aβ interacts with other receptors such as tyrosine kinase (TrkA), pan-neurotrophin p75 (p75NTR) and α7 nicotinic acetylcholine (nAChR) (129).

In response to Aβ-induced neurotoxicity in AD, both humoral and cellular immunity are activated. Generally, Aβ triggers microglia inflammatory cytokine production via toll-like receptors (TLR) and contributes to the enhancement of the inflammatory response by NF-κB stimulation, a nuclear factor that regulates mitogen-activated protein kinase (MAPK) pathways that lead to the production of proinflammatory mediators (130).

Aβ also plays a role in tau pathology. First, Aβ induces tau hyperphosphorylation by the activation of tau kinases; second, Aβ decreases tau degradation by the promotion of proteasome dysfunction; finally, Aβ activates caspase-3, which causes the truncation of tau and altered tau aggregation (117). Strategies that block these Aβ effects may prevent cognitive decline in Alzheimer's disease.

In cultured neurons, Aβ-induced toxicity involves apoptosis, oxidative stress, and inflammation (131-133). A growing body of research suggests that increased ROS production and oxidative stress-induced neuroinflammation are the leading causes of neurodegeneration, leading to mitochondrial dysfunction and neuronal apoptosis (134-136). Oxidative stress leads to neuroinflammation and neurodegeneration by activating stress-triggered signaling pathways, such as Jun N-terminal kinase (JNK) signaling, p38 signaling, and extracellular signal-regulated kinase (ERK) signaling, which are also involved in cellular inflammation and apoptosis (137-139). On the other hand, due to their amphiphilic properties, Aβ peptides are implicated to directly interact with the neuronal plasma membrane and affect its functioning, thereby initiating above-mentioned

pro-apoptotic signaling and inhibiting intracellular anti-apoptotic signaling, such as phosphatidyl inositol 3-kinase/protein kinase B (PI3K/Akt) pathway (140-142). Besides the role in neuronal survival, PI3K/Akt signaling pathway has also been suggested to play a pivotal role in GSK3β-mediated tau hyperphosphorylation, which is another pathological hallmark of AD (143). Aβ peptides have been found to directly induce hyperphosphorylation of tau in neurons (144-146).

2.3 *ω*-3 PUFA

2.3.1 *ω*-3 PUFA and obesity

Obesity is considered to be the most significant public health problems, whose prevalence is increasing dramatically in both developed and developing countries over the last 20 years. Obesity has been associated with an increased risk of developing dyslipidemia, insulin resistance, hypertension, diabetes mellitus, CVD, osteoarthritis, respiratory complications, and cancer (67). Notably, low-grade inflammation has been identified as a key factor in the development of obesity and obesity-related diseases (8). Enhanced inflammatory activity of adipose tissue in obesity attributes to macrophage infiltration and increased levels of circulating adipokines secreted by adipocytes, such as IL-6, TNFα, and MCP-1 (9).

Omega-3 PUFAs are essential fatty acids in animals since they cannot be synthesized de novo and are vital for normal metabolism. Therefore, ω-3 PUFAs need to be supplied by diet. Alpha-linolenic fatty acid (ALA), which can be found in plants, is the precursor of other ω-3 PUFAs, SDA, EPA, and DHA. SDA is also plant-sourced, while DHA and EPA are mainly found in fish and fish oil, known as marine-sourced fatty acids. Adequate fish intake in our diet provides an optimal supply of ω-3 PUFAs in our metabolism since in vivo conversion of ALA to DHA and EPA might be limited.

Long-chain PUFAs are known to participate in carbohydrate and lipid metabolism (147). In addition, long-chain PUFAs play an important regulatory role in immune responses and inflammation. As precursors of anti-inflammatory molecules (prostaglandins, leukotrienes, thromboxanes, lipoxins, and resolvins), ω-3 PUFAs are potent modulators of cytokine production (148). In particular, long-chain PUFAs, especially DHA and EPA, are incorporated into cell membrane phospholipids and converted through phospholipase A2, cyclooxygenase (COX) and lipoxygenase (LOX) to eicosanoids, such as prostaglandins, thromboxanes, leukotrienes, as well as various hydroxyl-fatty acids (10). In general, ω -3 PUFAs-derived eicosanoids have physiologically potent anti-inflammatory activity (10). Moreover, resolvins, protectins and maresins derived from DHA and EPA are discovered to be powerful anti-inflammatory agents, regulating the resolution of inflammation, as evidenced by inhibition of inflammatory cytokine production and reduction in leukocyte recruitment (149). On the other hand, the ω-3 PUFAs can compete for the same enzymes, COX and LOX, with the ω-6 PUFAs, which form a different class of eicosanoids with pro-inflammatory properties, thus can counteract the effects of the ω-6 PUFAs-derived eicosanoids (11).

The balance of ω-3 and ω-6 PUFAs is an important determinant in homeostasis maintenance, normal development, and mental health (150). However current data reveals the ω-6 to ω-3 ratio in Western diets as being between 8:1 and 17:1, which could promote obesity, inflammation, and related diseases, such as coronary heart disease, rheumatoid arthritis, diabetes, cancer, and mental illness (4). Previous studies suggested that plasma ω-3 PUFAs are negatively associated with obesity, whereas ω-6 PUFAs are positively associated with obesity (12, 13). Significantly increased ω-6 to ω-3 PUFAs ratio was reported in the liver of patients with non-alcoholic fatty liver disease (14). It was reported that a ω-6 to ω-3 PUFAs ratio of 4:1 was

associated with a 70 % decrease in total mortality of CVD; a ratio of 5:1 had a beneficial effect on patients with asthma; and a ratio of 2-3:1 suppressed inflammation in patients with rheumatoid arthritis (7).

Most of the beneficial effects attributed to ω-3 PUFAs have been observed after the consumption of DHA and EPA, and most data do not support the notion that ALA will provide the same physiological effects (4). Consumption of ω-3 PUFAs are associated with reduced body weight and fat mass. A lower incidence of CVD in Eskimos and Japanese has been reported to be associated with their increased plasma levels of EPA resulted from their eating habits-high intake of fatty fish (151). The substitution of visible fats with fish oil in diet has been demonstrated to reduce the body fat mass and stimulates lipid oxidation in healthy adults (152). Previous data indicated that higher consumption of fish and omega-3 PUFAs is associated with a reduced risk of thrombotic infarction, primarily among women who do not take aspirin regularly (153). The inclusion of either lean or fatty fish, or fish oil as part of an energy-restricted diet could result in approximately 1 kg more weight loss after 4 weeks, than did a similar diet without seafood or supplement of marine origin, in young and overweight men (15). In addition, consumption of ω-3 PUFAs are associated with an improvement of lipid profile and other metabolic syndrome features. Fish or fish oils were reported to decrease plasma triacylglycerol and increase HDL cholesterol in insulin-resistant obese men (16) an in overweight hypertensive subjects (17). In hypertriglyceridemic men, DHA supplementation could improve cardiovascular health by lowering concentrations of triacylglycerol and small, dense LDL particles (154). It has been found that the levels of long-chain ω-3 PUFAs, especially EPA, were associated with lower serum TG concentration in people with metabolic syndrome (155). Supplementation of moderate fish oil has been demonstrated to reverse low-platelet and reduce plasma TG levels in British Indo-Asians

(156). Moreover, consumption of ω-3 PUFAs are associated with an inhibition of insulin resistance and inflammatory state. The insulin sensitivity of patients with non-insulin-dependent diabetes has been found to be improved by dietary supplementation of ω-3 PUFAs (18). Use of cod liver oil during the first year of life has been shown to reduce the risk of type 1 diabetes, which might be associated with the anti-inflammatory effects of long-chain ω-3 PUFAs (157). In healthy older adults, high consumption of ω-3 PUFAs could increase insulin sensitivity and reduce inflammatory markers, CRP and IL-6 (19).

The effectiveness of DHA and EPA on cell function are different (158). Both DHA and EPA were shown to be able to decrease TG levels, but only DHA increased HDL and LDL particle size (159). Besides, DHA has been shown to be more efficient in decreasing blood pressure, heart rate and platelet aggregation compared to EPA (160).

For the recommended intake of ω-3 PUFAs, a consumption of two to three servings per week of fish rich in ω-3 PUFAs is suggested since it should provide about 500 mg/day of DHA and EPA for primary prevention of CVD in adult general population (4). For secondary prevention, patients with documented CHD should consume approximately 1 g EPA + DHA/day from oily fish or fish oil capsules (5). For quality in DHA and EPA supplements, determination on polychlorinated biphenyls, heavy metals (lead, cadmium, mercury, and arsenic) and oxidation levels, as well as purity and total amount of DHA and EPA are needed according to the Council for Responsible Nutrition (2006).

In conclusion, it can be suggested that supplementation of ω-3 PUFAs could be effective in improving obesity and metabolic syndrome symptoms; and most probably by modulating fat oxidation and satiety; however, mechanisms underlying these effects remain a challenging point still to be addressed in future. Oral supplementation with ω-3 PUFAs seems to be an effective way

to improve lipid profile, such as reduced plasma TG and increased HDL-cholesterol, which can be applied in metabolic diseases. The effectiveness of the ω-3 PUFAs might be related to dietary habits, exercise patterns, and gender aspects.

2.3.2 *ω*-3 PUFA and AD

Over the past 30 years, the health benefits of ω-3 PUFAs have been extensively explored. In addition to their known protective effects on heart disease (3), increased consumption of ω-3 PUFAs from fish oil have been implicated in reductions in cancer (161), inflammation (162), obesity (163), T2DM (164), and neurological disorders (165), such as AD. AD is now recognized as type 3 diabetes since an association between diabetes and increased risk of AD has been demonstrated (20). Present evidence suggests that onset of cognitive impairment in AD could have major underlying processes that cause neuronal death, including defective antioxidant defense mechanisms, inflammatory processes, and reduced mitochondrial energy production. Therapeutic interventions to delay or prevent the onset of cognitive impairment need to be explored. Long-chain ω-3 PUFAs have been found to exert beneficial effects on cognitive function in patients with early onset mild cognitive impairment (21). While, intake of saturated fatty acids have been found to induce cognitive decline (22). Minimizing consumption of saturated fats and trans-fats has been listed in the guidelines for the prevention of AD (23).

The beneficial effects from ω-3 PUFA consumption appear to be mediated through DHA and EPA. Cold water fish and fish oils are the most direct source of DHA and EPA. DHA and EPA can also be synthesized from ALA, but with limited conversion inefficiency (166). This suggests a minor role for ALA in reducing AD risk and, therefore, the emphasis of our review is on DHA and EPA omega-3 fatty acids. Notably, the long-chain ω-3 PUFAs are concentrated in

the phospholipid membrane of the brain, particularly at the synapses (167), and brains of AD patients have lower DHA. Lower contents of ω-3 PUFAs have been measured in the plasma and brain of patients with AD compared with normal healthy individuals (24-28). Normal physiological changes to the brain with aging include depletion of major long-chain ω-3 PUFAs, which is accelerated in neurodegenerative conditions such as AD (29).

Epidemiological observations suggest that high ω-3 PUFA intake from fatty fish or fish oil is associated with decreased risk of mild cognitive impairment, but not AD. To date, controlled studies conducted on patients with mild cognitive impairment and supplemented with ω-3 PUFAs suggest a positive effect on cognitive performance following supplementation ranging from 3 to 12 months (168). The Mediterranean diet with a healthier balance between ω-3 and ω-6 fatty acids, was reported to be associated with lower AD risk (169). Kotani and colleagues demonstrated that supplementation with 240 mg/day of DHA and 240 mg/day AA significantly improved immediate memory and attention scores in adults with mild cognitive impairment (30). The CHAP study from Chicago observed a reduced decline in global cognition over 6 years in 3700 participants (> 65y) who had at least 1 fish meal per week (170). Chiu and colleagues also reported improvements in patients with mild cognitive impairment following ω-3 PUFA supplementation over 24 weeks (171).

In animal studies, the beneficial effects of ω-3 PUFA were much more significant and consistent, and were observed particularly on Aβ deposition, tau pathology, neuron loss, and cognitive deficits. Ikemoto and colleges found that the altered learning behavior associated with a long-term ω-3 PUFA deficiency could be reversed by supplementing the diet with DHA-enriched oil (31). Wu and colleges demonstrated that the EPA-enriched phospholipids administration prevented the development of learning and memory impairments in SAMP8 mice, and suggested

that this neuroprotective effect may rely on the anti-oxidative activity and inhibition of mitochondria-dependent apoptotic pathway of EPA (32). Although the molecular basis for such protective effects of ω-3 PUFAs remains unclear, conclusion from the investigations in animal models and cell culture usually described to rely on 5 distinct and interconnected molecular mechanisms (33): (i) regulation of gene expression for various proteins involved in Aβ production and tau pathology (34), (ii) reduction of oxidative damage (32), (iii) block of inflammatory cascade (35), and (iv) inhibition of cell apoptosis pathways (36), as well as (v) membrane remodeling (37).

DHA, which is selectively enriched in neuronal tissues especially in neuronal and synaptic membranes (172) is most studied in ω-3 PUFAs in AD. Dietary intake of DHA is required for normal neurodevelopment and brain health, particularly during prenatal brain development (173). DHA has been shown to have a crucial role in regulating neural gene expression. DHA acts as an endogenous ligand for retinoic acid receptors (RAR) and retinoid x receptors (RXR), which have been shown to decrease with age and age-related memory deficits (174). Restoration in the decrease of RAR and RXR associated with memory deficits and increase in neurogenesis have been shown following DHA supplementation (174). In addition, it has been demonstrated that DHA facilitates the activation of protein kinase B (PKB), also known as Akt, via an increase in phosphatidylserine; activation of Akt can cause an increase in the levels of hippocampal BDNF, which further strengthens synaptic plasticity and neuronal survival. (175). Moreover, a diet rich in DHA has been found to activate Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), which signaling cascade is critical for induction and maintenance of long-term potentiation in hippocampus (176, 177). Further, DHA has an influence on membrane fluidity. By regulating the membrane fluidity, DHA is found to facilitate N-methyl-D aspartate (NMDA) responses and block K^+ channels, which results in long-term potentiation (178, 179). DHA has also been shown to

modulate gene expression at the transcription level by activating peroxisome proliferator-activated receptor (PPAR) family members (180). On the other hand, by regulating the membrane fluidity, DHA has been demonstrated to shift APP towards non-amyloidogenic processing, thereby reducing Aβ release (181).

2.4 Stearidonic acid

2.4.1 The advantage of SDA

Present evidence has indicated that a high consumption of the long-chain ω-3 PUFAs, DHA and EPA lowers CVD morbidity and mortality (182), and improves inflammatory conditions such as arthritis (183). However, as the major dietary source of DHA and EPA, oily fish consumption in Western countries including the United States remains low (38). Many individuals cannot tolerate the taste or smell of oily fish or fish oils, even when provided in capsules (39). In addition, yields from global fisheries have been reported to be stagnant or declining (40). Besides, there is an increasing alarm over levels of methyl mercury in some species of long-lived fish (41). Hence, there is a need and desire to identify and develop alternative sources of DHA and EPA that have similar biological properties. Alpha-linolenic acid (ALA; 18:3; ω-3) is the main ω-3 fatty acid available in vegetable oils, such as flaxseed oil, canola oil, and soybean oil. Normally, dietary ALA goes through the reactions of desaturation and elongation to convert to DHA and EPA. However, because of the rate-limiting enzyme-Δ6 desaturase, the conversion from ingested ALA to DHA and EPA is so poor that currently there is insufficient evidence that ALA has health benefits similar to DHA and EPA (42). Present evidence suggests that another plant-based ω-3 fatty acid, SDA, is more efficiently converted to EPA in the body than ALA (184). The lower unsaturation index of SDA greatly enhances its stability and shelf life with a concomitant reduction

in off flavors and odors that result from the generation of oxidative products (185). Therefore, SDA has been incorporated into a wide range of food products by using either oil or flour modified genetically from soybeans with enhanced levels of SDA. These foods range from oil-based products (SDA-soybean oil, salad dressing) to dairy products (milk, cheese) to prepared foods (energy bars).

2.4.2 SDA metabolism

SDA is a PUFA with an 18-carbon chain and four double bonds in the acyl chain. Its molecular weight is 276.4, and its melting point is -57 ºC (186). SDA lies in the biosynthetic pathway of the EPA, DPA, and DHA from ALA, as the intermediate between ALA and EPA. ω-3 fatty acid metabolic pathway involves a series of desaturation, elongation, and oxidation reactions, with the rate-limiting enzyme Δ6 desaturase (187). The gene expression of the Δ6 desaturase is downregulated by increasing age, the presence of certain hormones, insulin resistance, and in response to ω-3 fatty acids (188).

2.4.3 Dietary source of SDA

SDA is found in the seeds and leaves of several plants, including those of the boragenase family, such as echium plantagineum, borage (*boragoofficinalis*), evening primrose (*oenotherabiennis*), and blackcurrant (*ribesnigrum*). However, these plants are not adapted to wide scale cultivation, and oil yields are low and variable. Some individual foods have also been identified to be comparatively high in SDA content, including hemp oil, echium oil, borage oil, fish and other seafood. The SDA content of these seed oils is around 15% to 22% of total fatty acids (186). Fish oils also contain little SDA, which contributes approximately 0.5-4% of the total fatty acids (189, 190). Echium oil, as a natural source enriched in SDA, which account for

approximately 12% of total fatty acids in the oil is most studied in animal models (191). SDA-enhanced soybean oil was created by the introduction of Δ6 desaturase from primrose (*primula juliae*) and Δ15-desaturase from red bread mold (*neurospora crassa*) (192). Genetic-modified SDA-enriched soybean oil usually to around 18%-28% of total fatty acid content (193). SDA-enriched soybean oil is now suggested to serve as a convenient vehicle for increasing the dietary intake of this "pro-EPA" fatty acid (193). Very recently, ahiflower oil has been found to be the richest natural source of SDA (20%) in Canada (194).

2.4.4 Effects of SDA on the Omega-3 index

Omega-3 index is the sum of DHA and EPA in the membrane of red blood cells and expressed as a percentage of total erythrocyte fatty acids. Omega-3 index has been proposed as a risk factor for CVD (195). It has been demonstrated that dietary vegetable oils containing SDA was more effective in increasing EPA concentrations in erythrocyte and in plasma phospholipids than are current ALA-containing vegetable oils (196). This result was verified by a randomized controlled trial of healthy subjects with overweight or obese, SDA intake of 3.7 g/d as SDA-enriched soybean oil significantly increased the levels of omega-3 index in red blood cells (197). In particular, SDA increased red blood cell EPA by approximately 17%, whereas the efficiency of ALA was approximately 0.1% (197). Another study further verified that dietary intake of SDA-enriched soybean oil increased the omega-3 index by raising erythrocyte EPA concentrations, but not DHA concentration (198). Higher omega-3 indexes have been associated with a reduction in risk for sudden cardiac death (195), therefore, the impact of SDA intake on the omega-3 index may suggest its beneficial effect on CVD.

2.4.5 SDA as a feed component

SDA has been studied as an animal feed component to enrich the long-chain ω-3 PUFA content of meat, fish, and milk. In dairy cows, feeding with SDA increased the EPA content in plasma and milk (199). In lambs receiving an abnormal infusion of echium oil, muscle tissue levels of EPA were increased approximately 3-4 fold after 4 weeks compared with those infused with saline (200). In sows and piglets, dietary intake of echium oil significantly increased the EPA content of plasma (201). In dogs, dietary SDA consumption significantly increased the red blood cell and heart EPA content, and the efficacy was 20-23% of that by EPA treatment (202). In broiler chickens, feed SDA-enriched oils led to significant increase of EPA content in egg yolk, liver, and meat of chickens (203, 204).

2.4.6 Bioactive effects of SDA

In rodent study, dietary SDA intake resulted in significant increase of EPA levels in red blood cells, muscle, liver, ileum, and brain (45, 46, 48, 205-207). In humans, intake of SDA as SDA ethyl ether, echium oil, SDA-enriched soybean oil or other known sources, has been shown to significantly increase concentrations of long-chain ω-3 fatty acids in blood and tissues (184, 191, 196, 208). In the brains of neonatal rats, the content of long-chain ω-3 PUFAs was found to be increased without elevating hepatic lipid concentration by echium oil diet (209).

Clinical and experimental studies indicate that dietary SDA shares many of the biological effects of long-chain ω-3 PUFAs. Recently, several studies established the emerging roles of SDA in inflammation (48, 210-213), dyslipidemia (45, 191, 207, 209, 214, 215), atherosclerosis (46), CVD (197, 208, 215), and cancer (216-218), suggesting SDA could become a new supplemental source of ω-3 PUFAs in health promotion and disease prevention. In a study by Kuhnt and colleges

(208), normal or overweight healthy humans were instructed to consume SDA (2g/d) in the form of echium oil. After 8 weeks, metabolic markers, such as serum cholesterol, low density lipoprotein (LDL)-cholesterol, oxidized LDL and TG, decreased significantly compared with baseline. Similar results were shown in the study conducted by Surette and colleagues (191), in mildly hypertriglyceridemic subjects, plasma TG were lowered by an average of 21% compared with baseline by taking echium oil. In LDLr knockout mice with hypertriglyceridemia, a diet containing echium oil, comparable to that of fish oil, significantly decreased plasma and/or liver TG, total cholesterol and very low density lipoprotein (VLDL) lipid concentration compared to the control (45, 46, 207). Similar results were obtained in obese Zucker rats (215) and normal healthy rats (214), both fed by SDA-soybean oil.

When patients with mild asthma were supplemented with combination of borage oil and echium oil, the ex vivo leukotriene generation in stimulated basophils and neutrophils were significantly attenuated (211). In a mouse ear inflammation model, SDA and EPA isolated from the seaweed *Undariapinnatifida* showed inhibitory effects against the inflammatory symptoms of edema, erythema, and blood flow (219). In addition, SDA can mimic EPA in reducing inflammatory cytokines TNFα and PGE2, released from lipopolysaccharide (LPS)-stimulated whole blood and splenocytes of Balb/c mice (48), or released directly from intestinal production in Balb/c mice (213). This effect may be dependent on the suppression of COX-2, which is responsible for formation of prostanoids, including prostaglandins, prostacyclin and thromboxane (220). In the adipose stem cells, SDA was found to inhibit the LPS-induced IL-6 secretion and IL-6 mRNA expression by decreasing toll-like receptor-2 (TLR2)-mediated NFκB signaling pathway (210). SDA was also found to effect p38 MAPK activity in *Caenorhabditis elegans* (221). Kavanagh and colleagues (222) assessed the effect of SDA on glucose disposal in insulin resistant

monkeys. In this study, dietary echium oil significantly improved the ability to remove blood glucose from the circulating in monkeys after glucose challenge when compared to safflower oil and fish oil groups.

Chapter 3 Suppression of adipocyte differentiation and lipid accumulation by stearidonic acid in 3T3-L1 cells.

3.1 Abstract

Obesity is a complex disorder involving an excessive amount of body fat and has become one of the most serious health problems with the increasing prevalence in global. Long-chain ω-3 PUFAs, mainly EPA (C20:5; ω-3) and DHA (C22:6; ω-3), have been widely reported to protect against the development of obesity and reduce body fat. However, due to concerns regarding the fish palatability, global stagnant fishery industry, and possible methyl mercury contamination in some fish species, there is a need and desire to identify and develop alternative sources of DHA and EPA that have similar biological properties.

As the precursor of EPA in the ω-3 fatty acid metabolic pathway, SDA has been targeted as a potential biologically active surrogate for EPA in fish oil due to its efficient conversion following consumption. The purpose of this study was to evaluate whether SDA will affect adipocyte differentiation and lipid accumulation, which have been seen in DHA and EPA treatment. By fatty acid analysis, we found that SDA effectively incorporated into 3T3-L1 adipocytes and successfully converted to EPA. Specifically, cellular content of EPA was increased by treatment of 50, 100, and 200 μM SDA by 191%, 345%, and 537%, respectively compared with the control group. The effectiveness of EPA enrichment in 3T3-L1 adipocytes by SDA was approximately 35% of that by EPA treatment. However, SDA didn't affect the DHA levels in 3T3-L1 adipocytes.

Next, we measured lipid accumulation in 3T3-L1 adipocytes affected by ω-3 fatty acids. By Oil Red O staining, we found that treatment of 50 μM DHA, EPA, and SDA significantly

reduced the accumulated lipid droplets in 3T3-L1 adipocytes by 12%, 12%, and 8%, respectively. Treatment of 200 μM ALA, DHA, EPA, and SDA significantly reduced the lipid content by 6%, 20%, 22%, and 14%, respectively. By triglyceride assay, we demonstrated that treatment of 50 μM DHA, EPA, and SDA significantly decreased the accumulation of TG by 31%, 30%, and 15%, respectively. Treatment of 200 μM DHA, EPA, and SDA significantly decreased the TG content by 59%, 54%, and 47%, respectively. In addition, by quantitative real-time PCR (qRT-PCR), we further demonstrated that the anti-adipogenic effect by SDA may rely on its down-regulation of mRNA levels of the adipogenic transcription factor, SREBP-1c and the lipid accumulation genes, AP2, FAS, SCD-1, LPL, GLUT4, and PEPCK. In summary, we demonstrated that SDA is able to suppress the adipocyte differentiation and reduce lipid accumulation in 3T3-L1 adipocytes by affecting the expression of associated genes. Our findings warrant further study to develop SDA as a natural and effective surrogate for EPA in the prevention or treatment of obesity.

3.2 Background

Obesity is a complex disorder involving an excessive amount of body fat and has become one of the most serious health problems with the increasing prevalence in global. It is estimated that approximately 2.3 billion adults are considered to be overweight and at least 700 million are obese in 2015 according to World Health Organization (223). Aside from being a social stigma, overweight or obesity is also recognized as an important risk factor in the development of various health complications, such as type 2 diabetes, hyperlipidemia, CVD, and certain types of cancer (224, 225). Research on how to prevent and treat obesity is an area of great interest. Although pharmaceutical interventions and surgical options have been adopted to address obesity, the proper balanced dietary habit is always the primary strategy. Great attention has been focused on ω-3

PUFAs, mainly EPA (C20:5; ω-3) and DHA (C22:6; ω-3), which have been reported to protect against the development of obesity and reduce body fat (226, 227). Cold water fish and fish oils are the most direct source of DHA and EPA. However, many individuals cannot tolerate the taste or smell of oily fish or fish oils, even when provided in capsules (39). In addition, yields from global fisheries have been reported to be stagnant or declining (40). Besides, there is an increasing alarm over levels of methyl mercury in some species of long-lived fish (41). Hence, there is a need and desire to identify and develop alternative sources of DHA and EPA that have similar biological properties.

ALA (18:3; ω-3) is the main ω-3 fatty acid available in vegetable oils, such as flaxseed oil, canola oil, and soybean oil. Normally, dietary ALA goes through the reactions of desaturation and elongation to convert to DHA and EPA (Figure 1). However, because of the rate-limiting enzyme-Δ6 desaturase, the conversion from ingested ALA to DHA and EPA is so poor that currently there is insufficient evidence that ALA has health benefits similar to DHA and EPA (42). SDA (18:4; ω-3), as a metabolic intermediate between ALA and EPA, represents the Δ6 desaturation product of ALA, and thus bypasses the rate-limiting step in the conversion of dietary ALA to DHA and EPA. Due to its relatively efficient conversion following consumption, SDA has been targeted as a potential biologically active surrogate for EPA (228). SDA is found in the seeds and leaves of several plants, including those of the boragenase family, such as echium plantagineum, borage (*boragoofficinalis*), evening primrose (*oenotherabiennis*), and blackcurrant (*ribesnigrum*). However, these plants are not adapted to wide scale cultivation, and oil yields are low and variable. That's why the genetic-modified SDA-enriched soybean oil has become the most common source of SDA with a content around 20-30% (44). Very recently, ahiflower oil has been found to be the richest natural source of SDA (20%) in Canada (194). Studies have shown that SDA was able to

improve lipid profile (45, 46, 191, 206-208, 215, 229), decrease leukotriene generation (47), inhibit inflammation (48, 220, 230), increase cardiovascular risk marker omega-3 index (49, 197), attenuate hepatic steatosis (45), reduce atherosclerosis (46) and slow cancer growth (50, 231, 232).

Obesity is characterized at the cellular level by increase in the number and size of individual adipocyte. The size of adipocyte increases because of increased storage of lipid, mainly TG from dietary sources or endogenous lipogenic pathways. The number of adipocyte increases as a result of increased proliferation and differentiation. These processes are known as adipogenesis, which has been extensively studied in 3T3-L1 cell line. Normally, 3T3-L1 preadipocytes can be induced to differentiate into mature fat cells under the control of a coordinated adipogenic gene expression, which is mediated by a number of transcription factors. Specifically, 3T3-L1 adipogenesis is triggered by treating the cells with insulin, DEX and isobutylmethylxanthine (IBMX). Upon the initiation, several transcription factors, such as CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor (PPAR)γ, and sterol regulatory element-binding protein (SREBP)-1c are activated to stimulate the expression of genes involved in lipid accumulation, such as AP2, FAS, SCD-1, LPL, GLUT4, and PEPCK (233). These genes involved in lipid metabolism further mediate cellular uptake of fatty acids and glucose, TG hydrolysis and lipogenesis, thereby leading to the characteristic phenotype of mature adipocytes. Supplementations with DHA and EPA had a beneficial effect on obesity associated with their impact on the metabolism of adipose tissue (234). In vitro studies revealed that DHA and EPA are able to inhibit adipocyte differentiation and decrease lipid accumulation by down-regulating the expression of certain transcriptional factors or lipolytic genes, such as C/EBP, PPARγ, SREBP-1c, AP2, FAS, SCD-1, and GLUT4 in 3T3-L1 adipocytes (235-240). However, the effect of SDA on adipogenesis is unknown. Therefore, the present study

hypothesized that SDA, as a metabolic surrogate for EPA, will suppress adipocyte differentiation and reduce fat deposition in 3T3-L1 cells through alterations in the expression of genes connected with lipid metabolism.

3.3 Materials and methods

3.3.1 Cell culture

3T3-L1 mouse embryo fibroblasts were purchased from American Type Culture Collection (ATCC Manassas, VA) and cultured in humidified atmosphere of 5% CO2, 95% air at 37 °C. The cells were maintained in a growth medium containing the following components: Dulbecco's modified Eagle's medium (DMEM) with high glucose, 10% fetal calf serum, and 1% penicillin-streptomycin. 3T3-L1 cells were differentiated into adipocytes as previously described (241). Two days after the cells reached confluence (Day 0), differentiation to adipocytes was initiated by incubating the cells in differentiation medium containing the following components: DMEM with high glucose, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.5 mM IBMX, 1 µM DEX and 10 µg/mL insulin. After three days (Day 3) fresh differentiation medium containing only insulin was added for further two days. On Day 5 medium was replaced back to growth medium until sample collection. All cell culture components were purchased from Sigma (St. Louis, MO).

3.3.2 Fatty acid treatment

Fatty acids (ALA, SDA, EPA, and DHA) were purchased from Matreya LLC, (Stage College, PA). Stock solutions of fatty acids were in ethanol and further diluted in DMEM containing 1.5% of fatty acid-free bovine serum albumin (BSA). Fatty acids were delivered to the cells as fatty acid/BSA complexes. BSA-ethanol vehicle was used a control. After incubation at

37ºC for 1h, fatty acid-supplemented medium (50 or 200 μM) or BSA-ethanol vehicle control was applied to 3T3-L1 adipocytes from Day 0. Cells were harvested on Day 6.

3.3.3 Fatty acid analysis

3T3-L1 cells differentiated for six days in the absence or presence of fatty acids were used for fatty acid analysis. Lipid extracts from 3T3-L1 adipocytes were prepared using chloroform/methanol (C/M, 1/1, v/v). The organic phase was collected, dried under N2 gas, and dissolved in C/M 1/1. Saponification and formation of fatty acid methyl esters made from cellular lipid was then performed for liquid chromatography/mass spectrometry (LC/MS). The instrument we used is Agilent 1290 UHPLC coupled Agilent 6460 QQQ triple quadruple mass spectrometer. LC/MS was conducted to quantify the content of DHA and EPA within adipocytes. Palmitic acid-d31 (Sigma, purity > 99%) was added as internal standard. Fatty acid content was normalized to the protein content. Protein quantification was performed using the Bio-Rad DC Protein Assay Kit (Bio-Rad, CA). BSA standard curve and sample preparation and analysis were realized according to manufacturer's instructions.

3.3.4 Oil Red O staining

3T3-L1 cells differentiated for six days in the absence or presence of fatty acids were washed with PBS and then applied to Oil Red O staining assay according to the protocol of a commercial kit (Abcam, #133102). Briefly, cells were first washed with PBS and fixed with formalin solution for 15 minutes. The fixed lipid droplets were then stained with Oil Red O solution for 30 min at room temperature. Microscope images were taken to visualize pink to red oil droplets staining in differentiated cells. For quantification of lipid content, the red oil dye was eluted with isopropanol solution and the absorbance was measured at 490 nm on a microplate

reader using the Bio-Tek spectrophotometer (Winooski, VT). The absorbance was normalized to the protein content.

3.3.5 Triglyceride accumulation assay

3T3-L1 cells differentiated for six days in the absence or presence of fatty acids were used to quantify TG accumulation with a commercial kit (Abcam, #102513). According to the protocol, cell monolayers were washed with PBS and intracellular lipid, including TG were extracted with lipid extraction solution under heating. The TG content were then determined by adding lipase, which converted TG to glycerol. Glycerol was subsequently oxidized to convert the probe to generate color, which can be measured at 570 nm. According to the standard curve made from TG standards, the TG concentration of each sample was calculated. TG level was normalized to the protein content.

3.3.6 Total RNA isolation and quantitative real-time PCR analysis

3T3-L1 cells differentiated for three or six days in the absence or presence of SDA (50 or 200 μM) were washed with PBS and total RNA was extracted using RNeasy Mini Kit (Qiagen; Valencia, CA) according to manufacturer's instructions. The quality and concentration of total RNA was determined spectrophotometrically using NanoDrop (Thermo Scientific). Complementary DNA (cDNA) was synthesized from 1μg of RNA using iScriptTM cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Reverse transcription was performed with sample incubation at 25ºC for 5 minutes, followed by 42ºC for 30 minutes and then 85ºC for 5 minutes. The synthesized cDNA was used immediately for real-time PCR or stored in a -20ºC freezer. Quantitative real-time PCR (qRT-PCR) was performed in the MyiQ single-color real-time PCR detection thermocycler (Bio-Rad) using iQTM SYBR® Green Supermix (Bio-Rad)

to evaluate gene expression. Mouse gene specific primers were designed from Primer Bank and constructed by Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA). Oligonucleotide sequences of the primers used for amplification are presented in Table 1. Reaction mixtures were incubated for an initial denaturation at 95ºC for 3 minutes followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 30 seconds, and 55ºC for 10 seconds. The cycle threshold (ΔCT) method was used to measure relative quantification of the target gene, where values were normalized to the reference gene, 36B4. Fold changes of gene expression were calculated by the 2-ΔΔCT method. The statistical analysis was based on ΔCT values.

3.3.7 Statistical analysis

All data are presented as mean ± SEM. The statistical significance of differences between groups was determined by one-way analysis of variance (One-way ANOVA) and Student's t-test (two-tailed). The results were considered to be significant when the value of P was < 0.05. Figures were produced by GraphPad PrismTM version 6.01 (GraphPad software, San Diego, CA).

3.4 Results

3.4.1 SDA converted to DHA and EPA in 3T3-L1 adipocytes

As mentioned, SDA bypasses the rate-limiting step in the conversion of dietary ALA to DHA and EPA. Human and animal studies have demonstrated that the consumption of SDA increased EPA levels in many tissues including adipose tissue (215, 242). Here, we examined the DHA and EPA enrichment affected by SDA treatment in vitro. 3T3-L1 preadipocytes were differentiated in the absence or presence of fatty acids for six days. As shown in Figure 2A, cellular content of EPA was increased in adipocytes treated with 100 μM of ALA, EPA, or SDA by 103%, 987%, or 345%, respectively compared with control. Specifically, SDA increased adipocyte EPA

content 3.5-fold more efficiently than comparable levels of ALA and was approximately one-third as effective as EPA. While, adipocyte DHA content was significantly increased by 100 μM treatment of DHA, EPA, or SDA by 1967%, 28%, or 10%, respectively (Figure 2B). Specifically, SDA increased adipocyte DHA 50% less efficiently than comparable levels of EPA and was approximately 0.5% as effective as DHA. The effect on DHA enrichment by SDA is subtle. However, EPA enrichment by SDA is effective and this result indicates that the effect of SDA on adipocyte differentiation and TG accumulation found in the present study may be caused by its downstream metabolite EPA. SDA has little effect on DHA enrichment in 3T3-L1 adipocytes. Subsequently, we examined the DHA and EPA enrichment by different concentrations (50, 100, or 200 μM) of SDA. SDA was found to increase EPA content in 3T3-L1 adipocytes in a dose-dependent manner (Figure 2C). Specifically, 50, 100, or 200 μM SDA significantly increased adipocyte EPA content by 191%, 345%, or 537%, respectively. While DHA content was barely affected by SDA treatment (Figure 2D). There results verified that SDA can be a surrogate for EPA, but may not for DHA.

3.4.2 SDA decreased the lipid accumulation in 3T3-L1 adipocytes

3T3-L1 adipocytes store lipid as lipid droplets, which can be stained with Oil Red O, so that we can see them under microscope and quantify the lipid content by spectral analysis. To test the effect of SDA on lipid accumulation, 3T3-L1 preadipocytes were stimulated to differentiate into adipocytes with fatty acid supplementations and Oil Red O staining was conducted at Day 6. The micrographs of fatty acid-treated 3T3-L1 cells at Day 6 were shown in Figure 3A. It was found that ω-3 fatty acids decreased the size and the number of observed intracellular lipid droplets (Figure 3A). The quantification of Oil Red O staining showed that 50 μM treatment of DHA, EPA, or SDA significantly decreased the lipid content in 3T3-L1 adipocytes by 12%, 12%, or 8%,

respectively (Figure 3B). In addition, treatment of 200 μM ALA, DHA, EPA, or SDA significantly reduced the lipid content by 6%, 20%, 22%, or 14%, respectively (Figure 3B). These results indicate that SDA (≥ 50 μM) is effective in decreasing lipid accumulation in adipocytes, although the effect maybe not as good as DHA and EPA.

3.4.3 SDA decreased TG content in 3T3-L1 adipocytes

As TG is the main component of lipid droplets in 3T3-L1 adipocytes, the observation of reduced lipid content was confirmed by TG assay. 3T3-L1 preadipocytes were stimulated to differentiate into adipocytes with fatty acid supplementations and TG assay was conducted at Day 6. As shown in Figure 4, treatment of 50 μM DHA, EPA, and SDA all significantly decreased the TG content in 3T3-L1 adipocytes by 31%, 30%, and 15%, respectively. The efficacy of reducing TG content by 50 μM SDA was about 50% of the comparable levels by DHA and EPA. In addition, treatment of 200 μM DHA, EPA, or SDA significantly decreased the TG content by 59%, 54%, or 47%, respectively (Figure 4). The efficacy of reducing TG content by 200 μM SDA was about 87% and 80% of the comparable levels by DHA and EPA, respectively. This result was consistent with our previous results from Oil Red O assay. It is indicated that SDA (≥ 50 μM) is effective in inhibiting TG formation in adipocytes. In addition, this effect by SDA was in a dose-dependent manner and very comparable to that of DHA and EPA.

3.4.4 SDA decreased the expression of lipid accumulation genes

In order to determine whether SDA inhibits adipocyte differentiation and lipid accumulation by regulating the expression of associated genes, we examined the expression of SREBP-1c, AP2, FAS, SCD-1, LPL, GLUT4, and PEPCK by qRT-PCR. As shown in Figure 5A, the expression of SREBP-1c was significantly decreased by 37% in 3T3-L1 cells treated with 200

μM SDA compared to control cells. SREBP-1c is implicated in stimulating endogenous PPARγ ligand production (243). PPARγ is the central transcriptional regulator of adipogenesis, during which PPARγ is induced to activate a number of genes involved in fatty acid binding, uptake and storage, including AP2, FAS, SCD-1, LPL, GLUT-4 and PEPCK (233, 244). Subsequently, as shown in Figure 5B, the expression of AP2 was significantly decreased by 29% in 3T3-L1 cells treated with 200 μM SDA compared to control cells. As shown in Figure 5C, the expression of FAS was significantly decreased by 73% and 87% in 3T3-L1 cells treated with 50 and 200 μM SDA, respectively compared to control cells. As shown in Figure 5D, the expression of SCD-1 was significantly decreased by 42% and 47% in 3T3-L1 cells treated with 50 and 200 μM SDA, respectively compared to control cells. As shown in Figure 5E, the expression of LPL was significantly decreased by 25% and 90% in 3T3-L1 cells treated with 50 and 200 μM SDA, respectively compared to control cells. As shown in Figure 5F, the expression of GLUT4 was significantly decreased by 50% and 68% in 3T3-L1 cells treated with 50 and 200 μM SDA, respectively compared to control cells. As shown in Figure 5G, the expression of PEPCK was significantly decreased by 18% in 3T3-L1 cells treated with 200 μM SDA compared to control cells. In addition, these down-regulation of gene expression by SDA was all in a dose-dependent manner.

3.5 Discussion

DHA and EPA have been found to reduce fat accumulation in 3T3-L1 adipocytes through regulation of the expression in genes associated with adipogenesis (238, 245-247). SDA has been targeted as a potential biologically active surrogate for EPA since SDA can be effectively converted to EPA by the body and shares many beneficial properties with EPA (43). In the present

study, we compared the effects of these fatty acids on the prevention of adipogenesis, lipid accumulation and on the expression of genes connected with adipocyte metabolism in 3T3-L1 cells. Since ALA is the precursor of ω-3 fatty acids in the metabolic pathway and can also be converted to EPA, we also compared the influence of SDA with ALA on adipogenesis and lipid accumulation in our experiment. The effects of DHA and EPA directly were also investigated. For the concentration of ω-3 fatty acid treatment was use 50, 100, and 200 μM were used in our experiments. Based on previous studies, 200 μM is within the safe concentration for DHA, EPA, and ALA treatment (248, 249). In addition, we previously demonstrated that 200 μM is also a safe concentration for SDA treatment by cell viability assay (data not shown). Therefore, the suppressive effects of SDA and other investigated ω-3 fatty acids on adipocyte differentiation and lipid accumulation found in this study was independent of non-specific toxicity.

SDA is the Δ6-desaturase product of ALA in the bioconversion of ALA to EPA. In humans, the conversion of ALA to EPA is in low amounts (less than 7%) and in even low amounts to DHA (less than 1%) due to the rate-limiting enzyme (250). Therefore, nutritional supplementation with ALA was not sufficient to induce long chain ω-3 PUFA accumulation (251). In the present study, we hypothesized that by skipping the rate-limiting step, the conversion of SDA to EPA would be more efficient than that of ALA to EPA. By LC/MS analysis of DHA and EPA enrichment, we demonstrated that SDA increased EPA content in 3T3-L1 cells by 345% compared to control. The efficacy of EPA enrichment by SDA was about 3.5-fold greater than comparable levels of ALA and is about one-third of the enrichment by EPA. On the other hand, both SDA and ALA, even EPA was not able to increase DHA content effectively in 3T3-L1 cells, although statistical significance was found in SDA and EPA groups. This is probably due to another rate limiting step converting docosapentaenoic acid (DPA) to DHA (42). These findings agree with those of

previous studies. Studies have shown that consumption of SDA as ethyl esters, echium oil, or SDA-soybean oil increased EPA levels in red blood cells (49, 197, 242, 252-255), peripheral blood mononuclear cell (254), plasma (47, 242, 256), and neutrophils (256). In animal study, feeding with SDA increased EPA content in plasma and milk of dairy cows (199), plasma and muscle of lambs (200), plasma of sows and piglets (201), red blood cells and heart of dogs (202), egg yolk, liver, and meat of chickens (203, 204), and red blood cells, muscle, liver, ileum, and brain in rodents (45, 46, 48, 205-207). The efficacy of SDA on EPA enrichment in different tissues ranges from 17% to 85% as much as the efficacy of EPA on EPA enrichment based on human studies (49, 197, 242, 253-255). Furthermore, the efficacy of SDA was found to be 1.9 to 4.3-fold as much as that of ALA on EPA enrichment in human studies (242, 252). Nearly all studies found no significant change in DHA content after SDA supplementation. Based on these findings, we may conclude that SDA consumption would be expected to confer the health benefits associated with consumption of EPA, but not DHA.

Adipogenesis and increased lipid accumulation are key features in obesity. Beneficial effects of DHA and EPA from fish oil against adipose tissue expansion or adipocyte differentiation were previously identified under various experimental conditions (226, 234). In the present study, 3T3-L1 cell line was select since it is one of the most well-characterized and reliable models of studying adipogenesis. Mature adipocytes are larger, round, and filled with large lipid droplets, which can be distinguished from fibroblast-like preadipocyte in morphology. It has been shown that the formation of lipid droplets was suppressed in 3T3-L1 adipocytes by EPA (245, 246) or DHA treatment (245). Consistent with previous results, treatment of SDA significantly reduced the lipid accumulation in 3T3-L1 adipocytes and this effect by SDA was observed similar to the effects by DHA and EPA.

TG detection assay was conducted to confirm the inhibitory effect by ω-3 fatty acids on lipid accumulation. We demonstrated that SDA decreased TG concentration in 3T3-L1 adipocytes, which was similar to DHA and EPA. This result is in line with previous studies. An in vitro study revealed that treatment with high concentrations of sea cucumber and blue mussel extracts, which were rich in DHA and EPA, led to decreased accumulation of TG in 3T3-L1 adipocytes (239). One recent study has shown that treatment of differentiating 3T3-L1 adipocytes with DHA and EPA resulted in decreased cellular TG content, while treatment of saturated fatty acids increased TG accumulation (257). In animal studies, it was reported that dietary SDA has a potent to lower serum TG levels. A recent study showed that intake of SDA-enriched soybean oil decreased serum TG level in male Wistar rats (229). Studies with LDLr knockout mice, a model of hyperlipidemia and atherosclerosis demonstrated that supplementation with echium oil improved the whole lipid profile of the investigated mice, including lower TG and very-low-density lipoprotein (VLDL) concentrations (207), attenuated hepatic steatosis (258), and reduced atherosclerosis (46). In addition, the TG-lowering effect by SDA in animals has been found to be comparable to that of fish oil (46, 207, 229, 258). On the other hand, SDA was also reported to be effective in lowering serum TG levels in human studies. In a study by Surette and colleagues (2004), mildly hypertriglyceridemic individuals were instructed to consume SDA (1.88 g/d) in the form of Echium oil. After 4 weeks, the serum TG concentrations of these patients were decreased by 21% in average compare with baseline (256). The study of Kuhnt and colleagues (2014) also revealed that dietary echium oil was able to reduce the serum TG level, which has been recognized as an important cardiovascular risk factor in human with slight metabolic syndrome (254). However, a few studies did not find any difference in serum TG level by SDA supplementation (252, 259). In the present study, lipid and TG formation are profoundly suppressed by ω-3 PUFAs in vitro. This

action is most evident in the long-chain and highly unsaturated DHA, followed by EPA, relative to SDA. To our knowledge, this is the first study showing the influence of SDA on lipid droplets and TG accumulation in adipocytes.

The differentiation of preadipocytes is regulated by a complex network of transcription factors, mainly C/EBP families and PPARγ. C/EBPβ is one of the first transcription factors induced during adipocyte differentiation that is required for the expression of downstream transcription factors-C/EBPα and PPARγ (233, 260). SREBP-1c is implicated in stimulating endogenous PPARγ ligand production (243). C/EBPα and PPARγ are the central transcriptional regulators of adipogenesis, during which C/EBPα and PPARγ are induced to activate a number of genes involved in fatty acid binding, uptake and storage, including AP2, FAS, SCD-1, LPL, GLUT-4 and PEPCK (233, 244). In vitro, DHA and EPA have been reported to reduce adipogenesis through the inhibition of adipogenic transcription regulators (C/EBPs, PPARγ, and SREBP-1c) and their downstream target genes (AP2, FAS, SCD-1, LPL, GLUT4 and PEPCK) (236, 238, 245-248, 251, 252, 257, 261-269). In vivo, Ping Zhang and colleagues. (2008) reported that dietary echium oil, containing 12.5% of SDA, has a similar effect to fish oil in reducing the expression of SREBP-1c, FAS, and SCD-1 in livers of mice compared to a palm oil diet (207). In our previous study, it was showed that SDA treatment significantly down-regulated the expression of C/EBPα, C/EBPβ, and PPARγ (data not shown). Therefore, in the present study, we further examined the effect of SDA on the expression of genes that are targeted by C/EBPs and PPARγ. As expected, we observed dose-dependently down-regulation of SREBP-1c, which is implicated in stimulating endogenous PPARγ ligand production (243). In addition, consistent with the suppression of these adipogenic transcription factors, the expression of AP2, FAS, SCD-1, LPL, GLUT4 and PEPCK were all significantly decreased by SDA treatment in 3T3-L1 cells. AP2 binds

to long-chain fatty acids and facilitates their uptake and transport through cellular membrane. FAS is a multi-enzyme protein that catalyze the synthesis of fatty acids. SCD-1 is responsible for the biosynthesis of monounsaturated fatty acid oleic acid and thus plays a key role in fatty acid metabolism. As a cell surface lipolytic enzyme, LPL mediates lipoprotein uptake for the maintenance of lipid droplets. Based on our results, it was demonstrated that ω-3 fatty acids exerted effects to suppress lipid droplet formation in part due to down-regulated gene expression of LPL, with a simultaneous suppression of the gene expression of SCD-1, whose expression was reported to positively correlate with lipid droplet formation (270). PEPCK converts oxaloacetate into phosphoenolpyruvate and promotes synthesis of glycerol-3-phospate, which is used for TG synthesis. GLUT4 uptakes glucose and helps with the energy storage in adipocytes. Since GLUT4 is also connected with glucose metabolism and insulin sensitivity; down-regulation of GLUT4 gene expression was also observed in 3T3-L1 adipocytes induced by pro-inflammatory adipokine IL-6; and DHA and EPA have been reported to have preventive properties in the development of insulin resistance (271, 272) and inflammation (273, 274), it is necessary to further investigate the effect of SDA on adipokine secretion and its role in insulin resistance and inflammatory response in adipocytes for future study. Taken together, down-regulation of these transcription factors and genes by SDA treatment would explain the observed negative effects of SDA on adipogenesis and lipid accumulation.

The anti-adipogenesis properties of ω-3 fatty acids, especially EPA may attribute to its influence on eicosanoid biosynthesis. EPA can incorporate into the membrane phospholipids, which may lead to competition with ω-6 PUFA arachidonic acid (AA), resulting in reduced level of the metabolites of AA and 2-series prostanoids (238, 268, 275). AA regulates adipogenesis oppositely with EPA. Presence of EPA during the initial period of differentiation might be

responsible for smaller lipid droplet formation, while presence of AA tended to deposit lipids in large form of lipid droplets (276). In addition, AA and its derivatives--prostaglandins, such as PGI2 and PGF2α, have been reported to be closely related with terminal differentiation of adipocytes (277, 278). Therefore, the influence of SDA on the expression of prostaglandins and the direct effect of these metabolites on adipocyte differentiation need to be studied in the future. Lipidomics, as a large-scale study of pathways and networks of cellular lipid metabolism is rapidly popular and can be used to study this area. In the present study, BSA existing in cell culture media containing FBS was used to deliver fatty acids to 3T3-L1 cells, which is different from the transport of fatty acids by lipoproteins as macromolecules in the blood. To address this question, the effect of SDA on adipose tissue mass and morphology in an animal model need to be studies.

3.6 Conclusions

In summary, we demonstrated that SDA is able to inhibit adipocyte differentiation and reduce lipid accumulation in 3T3-L1 adipocytes. The possible mechanism for this action might be converting to EPA and down-regulating the expression of genes associated with adipogenesis and lipid accumulation. The influence of SDA is dose-dependent and similar to that of DHA and EPA. Our findings warrant further study to develop SDA as a natural and effective agent for the prevention or treatment of obesity.

Table 1 Oligonucleotide primer sequences used in real-time PCR for adipogenic transcriptional factors and lipid accumulation genes.

Gene	Forward primer	Reverse primer
SREBP-1	5'-GATGTGCGAACTGGACACAG-3'	5'-CATAGGGGGCGTCAAACAG-3'
AP2	5'-AAGGTGAAGAGCATCATAACCCT-3'	5'-TCACGCCTTTCATAACACATTCC-3'
FAS	5'-GGCTCTATGGATTACCCAAGC-3'	5'-CCAGTGTTCGTTCCTCGGA-3'
SCD-1	5'-TTCTTGCGATACACTCTGGTGC-3'	5'-CGGGATTGAATGTTCTTGTCGT-3'
LPL	5'-GGGAGTTTGGCTCCAGAGTTT-3'	5'-TGTGTCTTCAGGGGTCCTTAG-3'
Glut4	5'-GTGACTGGAACACTGGTCCTA-3'	5'-CCAGCCACGTTGCATTGTAG-3'
PEPCK	5'-CAGGATCGAAAGCAAGACAGT-3'	5'-AAGTCCTCTTCCGACATCCAG-3'
36B4	5'-AGATTCGGGATATGCTGTTGGC-3'	5'-TCGGGTCCTAGACCAGTGTTC-3'

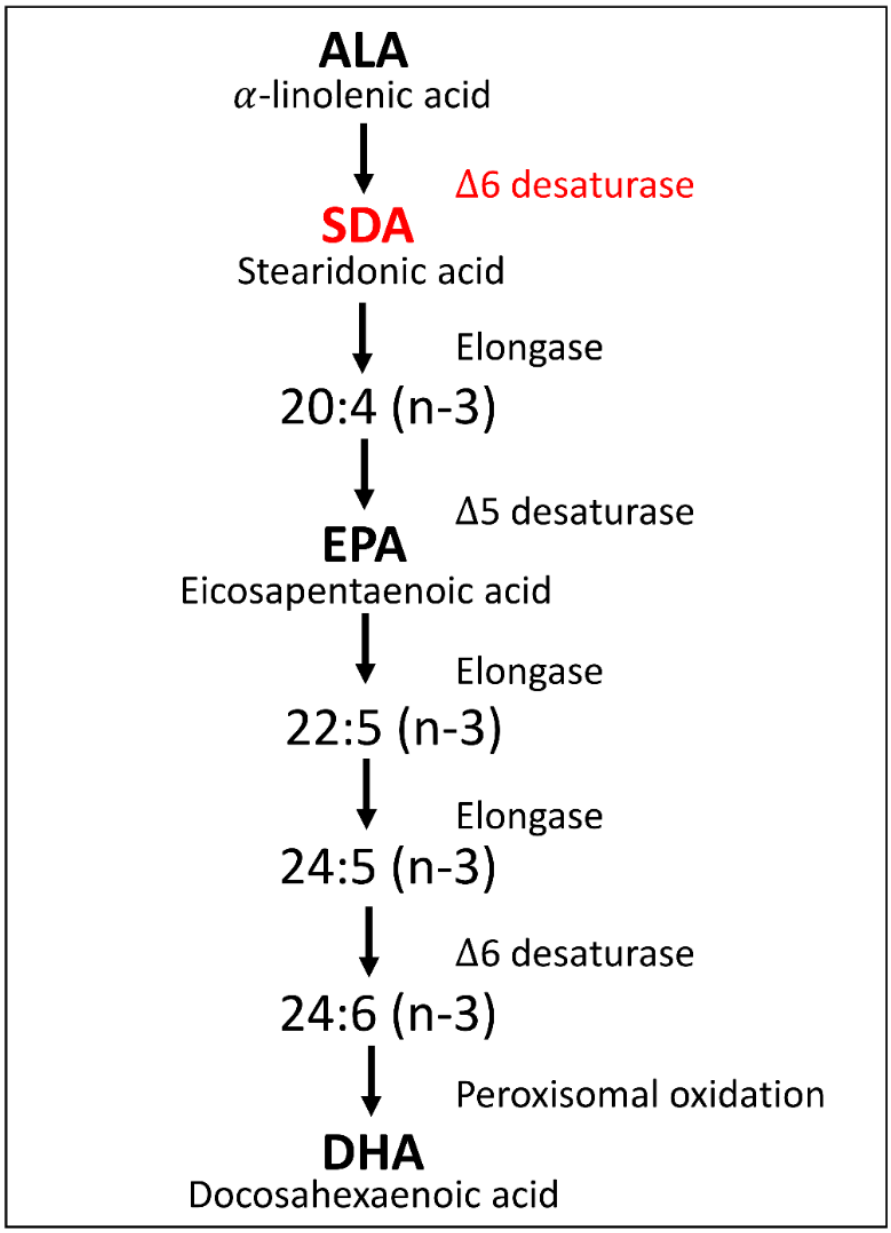

Figure 1 Metabolism of ω-3 fatty acids.

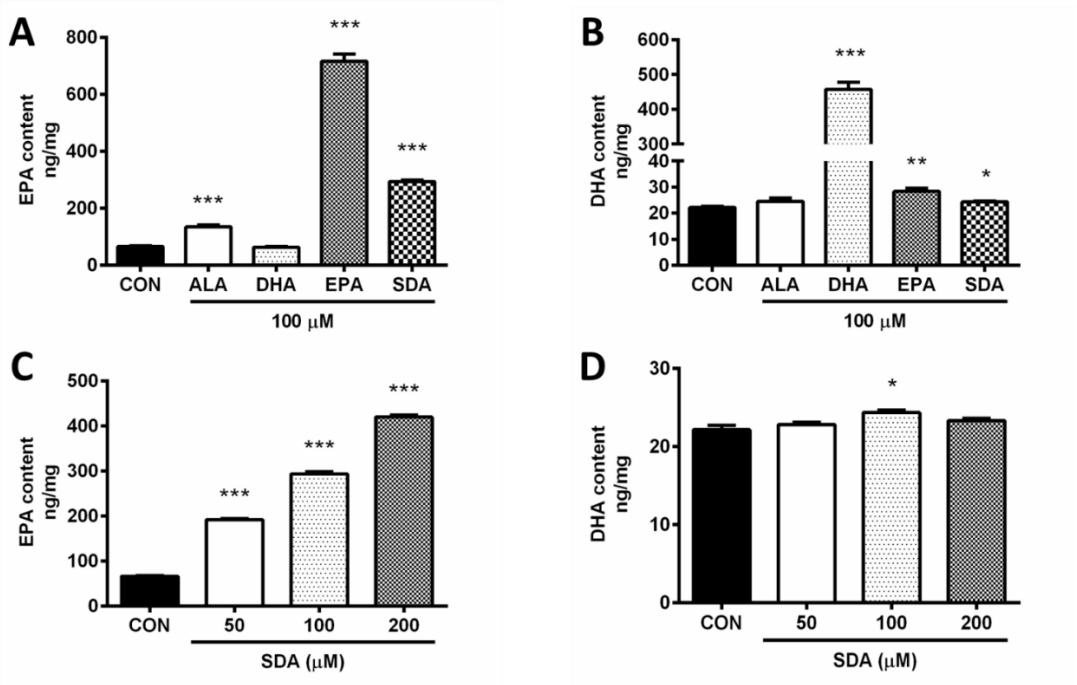

Figure 2 Enrichment of DHA and EPA by ω-3 fatty acid treatment in 3T3-L1 adipocytes.

Two-day post-confluence cells were incubated with differentiation medium containing different ω-3 fatty acids or BSA-ethanol vehicle for six days. EPA (A, C) and DHA (B, D) content was measured by LC/MS. Values were obtained from three independent experiments and are expressed as the means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

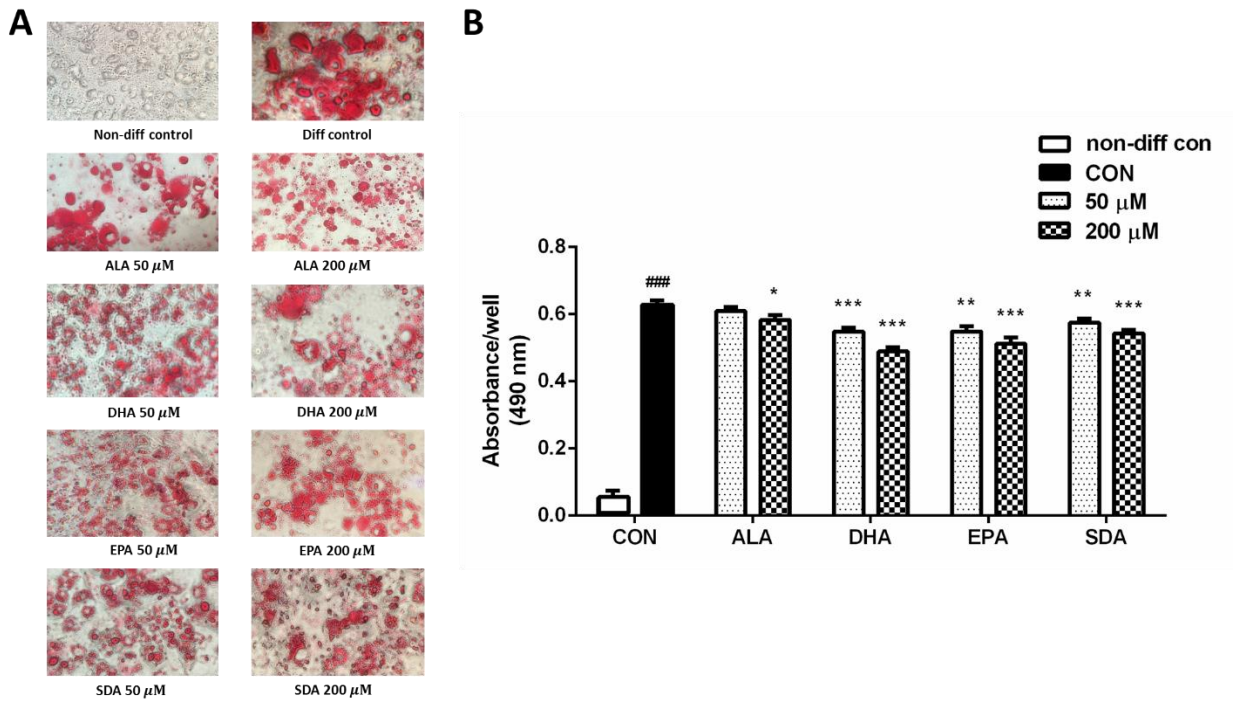

Figure 3 Effect of ω-3 fatty acid treatment on lipid accumulation in 3T3-L1 adipocytes.

Two-day post-confluence cells were incubated with differentiation medium containing different ω-3 fatty acids (50 or 200 μM) or BSA-ethanol vehicle for six days. Lipid content was measured by Oil Red O staining. (A) Representative images (200X) of Oil Red O staining. Lipid droplets were stained with red. (B) Intensities of Oil Red O staining measured by spectrophotometric analysis at 520 nm. Data were obtained from three independent experiments. Absorbance value is given as mean ± SEM; $^{###}P<0.001$, different from non-diff control; *P < 0.05, **P < 0.01, ***P < 0.001, different from differentiated BSA-ethanol vehicle treated control cells.

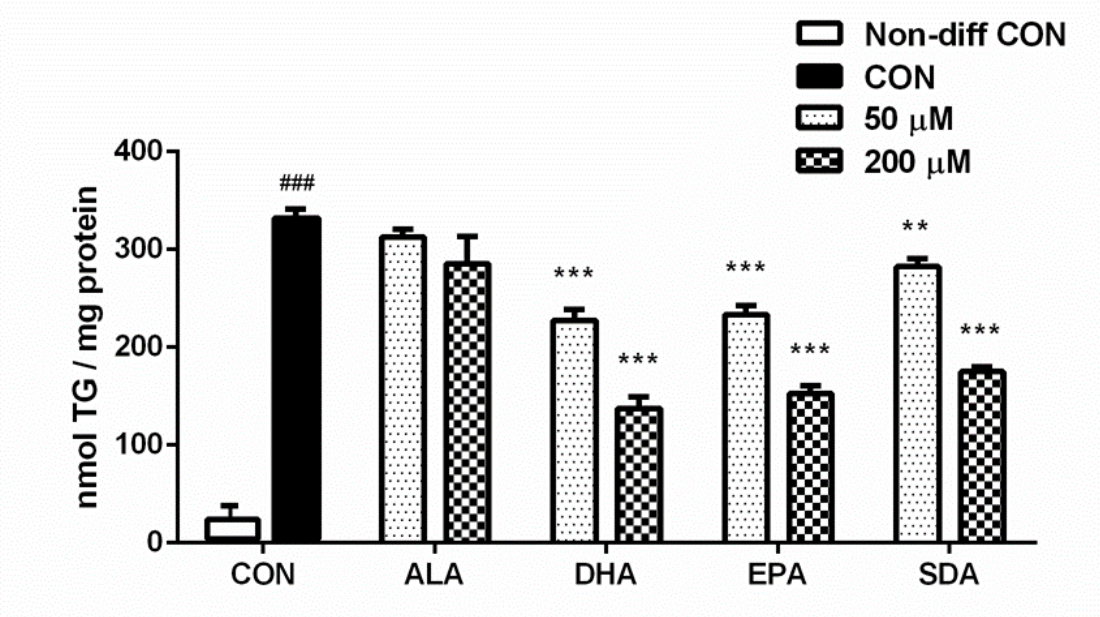

Figure 4 Effect of ω-3 fatty acids on TG content in 3T3-L1 adipocytes.

Two-day post-confluence cells were incubated with differentiation medium containing different ω-3 fatty acids (50 or 200 μM) or BSA-ethanol vehicle for six days. TG content was quantified by Adipogenesis Detection Kit (Abcam102512). Data were obtained from three independent experiments. Values are given as mean ± SEM; $^{###}P<0.001$, different from non-diff control; $^{**}P < 0.01$, $^{***}P < 0.001$, different from differentiated BSA-ethanol vehicle treated control cells.

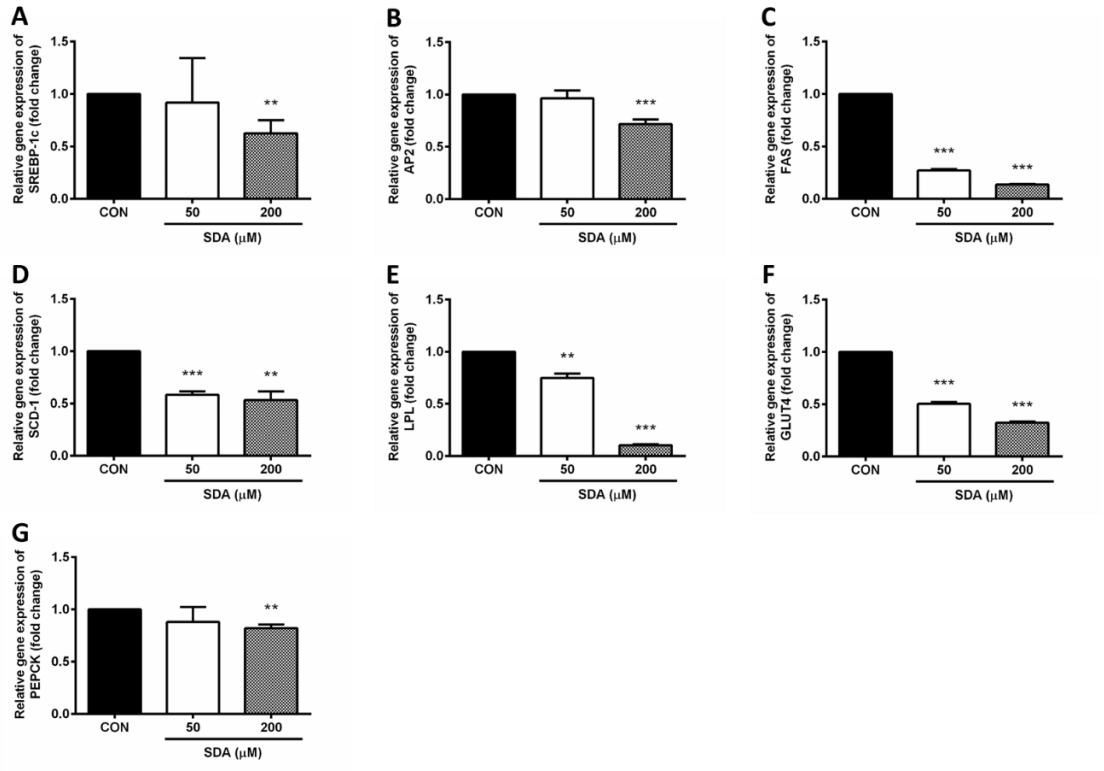

Figure 5 Effect of SDA on the expression of adipogenic transcription factors (A) and lipid accumulation genes (B-G).

Two-day post-confluence cells were incubated with differentiation medium containing SDA (50 or 200 μM) or BSA-ethanol vehicle for six days. The expression of SREBP-1c (A), AP2 (B), FAS (C), SCD-1 (D), LPL (E), GLUT4 (F), and PEPCK (G) were evaluated by qRT-PCR. Data were obtained from 3-4 independent experiments. Values were normalized to the level of 36B4 mRNA and are expressed as mean ± SEM; **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

Chapter 4 Neuroprotective effect of stearidonic acid against amyloidβ-induced neurotoxicity in H19-7 hippocampal cells

4.1 Abstract

AD is one of the major causes of dementia in the old population that involves an Aβ-induced cascade of an increase in apoptosis, oxidative stress, and inflammation. The present study demonstrated that the neuroprotective effects of SDA, a plant-sourced surrogate for long-chain ω-3 fatty acid, EPA, against 30 μM Aβ-induced apoptosis, oxidative stress, and inflammation in rat hippocampal cells. By fatty acid analysis, we demonstrated that SDA significantly incorporated into H19-7 hippocampal cells and successfully converted to EPA. Cellular content of EPA was increased by treatment of 50, 100, and 200 μM SDA by 65%, 121%, and 96% of the control cells, respectively. The effectiveness of EPA enrichment in H19-7 hippocampal cells by SDA was approximately 10% of that by EPA treatment. SDA didn't significantly affect the DHA level in H19-7 cells.

As the surrogate for EPA in neurons, pretreatment with 100 μM SDA significantly decreased neuronal cell death by increasing cell viability through a significant up-regulation of anti-apoptotic Bcl-2 and down-regulation of pro-apoptotic activated caspase-3, caspase-9, Bad, Bax, and Bik. SDA pretreatment improved the total anti-oxidant capacity of H19-7 cells through a significant up-regulation of anti-oxidant GPx-1, GPx-3, GSR, and SOD-1, and increasing the activity of anti-oxidant enzyme catalase, thereby mitigating Aβ-induced oxidative damage in H19-7 cells. In addition, pretreatment with 100 μM SDA significantly decreased the Aβ and LPS-induced expression of proinflammatory cytokines IL-1β and TNFα through downregulation of

gene expression of certain proinflammatory mediators, COX-2, MCP-1 and TLR4. Furthermore, SDA pretreatment decreased the Aβ-induced amyloid precursor protein (APP) gene expression, thereby inhibiting Aβ or APP-induced neurotoxicity. Finally, we demonstrated that the anti-apoptotic, anti-oxidant, and anti-inflammatory effects of SDA in H19-7 cells through an attenuation of Aβ-induced phosphorylation of mitogen-activated protein kinase JNK and p38, and an enhancement of ERK phosphorylation depressed by Aβ. Most importantly, we demonstrated that the efficacy of anti-AD effects by SDA is much more significant than that of ALA, and very comparable to that of DHA and EPA, though may not as good as DHA and EPA. These findings verified that SDA can be an effective surrogate for EPA, and suggests that SDA, similar to neuroprotective DHA and EPA, exerts protective effects on Aβ-induced neurotoxicity through its anti-apoptotic, anti-oxidant, and anti-inflammatory properties.

4.2 Background

AD is an emerging public health concern and one of the leading causes of death for the global aging population. Currently, over 34 million people are diagnosed with AD worldwide and the prevalence is anticipated to triple over the next 40 years (279). This represents a significant economic and emotional burden, not only to the health system, but also to the families of those affected and the individuals themselves (280). AD is a major chronic neurodegenerative disorder characterized by progressive neuronal death, loss of memory and impairment of higher cognitive functions (281). The neuropathological hallmarks of human AD brain are the presence of extracellular plaques composed of amyloid β (Aβ) and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein (282). Plaques and tangles modulate oxidative injury, inflammatory responses, and cell apoptosis (283). The exact etiology of AD is unknown

but overproduction of Aβ, exaggerated oxidative stress, and neuroinflammation are widely recognized in individuals with AD and thereby play important roles in modulating neuronal death (284-286). Despite progress in symptomatic therapy for AD, effective therapeutic approaches that interfere with AD are still unavailable. AD is most associated with aging, but also largely affected by dietary nutrition. It is thus essential to identify the nutritional biological factors that could modulate the AD progression. Considerable attention has been focused recently on the potential influence of ω-3 PUFA in the CNS on the development of AD (287). PUFAs are bioactive molecules with diverse physiological functions ranging from its contribution in cell culture to signal transduction (288). Amongst the ω-3 PUFAs, DHA and EPA from fish oil are characterized as neuroprotective antioxidants, which play vital roles in the production of pro-inflammatory cytokine in human neuronal cells (289, 290), as well as in animal models (291, 292) and human trials of AD (35, 293). Epidemiological studies indicated that increased consumption of DHA and EPA from fish oil or fatty fish are associated with reduced risk of human AD (294). For instance, van Gelder and colleagues examined cognitive decline over a 5-year period and reported that increase in fish consumption and DHA/EPA intake are both associated with reduction in cognitive decline (295). In studies of transgenic AD mouse models or aged animals, learning, reference and working memory performance was also found to be enhanced by supplementation with ω-3 fatty acids (296-301). Furthermore, the contents of ω-3 fatty acids, especially DHA, have been reported to be significantly decreased in the plasma and brain of patients with AD as compared to healthy controls, suggesting a possible role of ω-3 fatty acids in the intervention of AD (302). In the hippocampus of AD patients, brain DHA levels are reduced by as much as 50% compared to those in non-AD of the same age (303-305).

Cold water fish and fish oils are the most direct source of DHA and EPA. However, many individuals cannot tolerate the taste or smell of oily fish or fish oils, even when provided in capsules (39). In addition, yields from global fisheries have been reported to be stagnant or declining (40). Besides, there is an increasing alarm over levels of methyl mercury in some species of long-lived fish (41). Hence, there is a need and desire to identify and develop alternative sources of DHA and EPA that have similar biological properties. ALA is the main ω-3 fatty acid available in vegetable oils, such as flaxseed oil, canola oil, or soybean oil. Normally, dietary ALA goes through the reactions of desaturation and elongation to convert to DHA and EPA. However, because of the rate-limiting enzyme-Δ6 desaturase, the conversion from ingested ALA to DHA and EPA is so poor that currently there is insufficient evidence that ALA has health benefits similar to DHA and EPA (42). SDA (18:4; ω-3), as a metabolic intermediate between ALA and EPA, represents the Δ6 desaturation product of ALA, and thus bypasses the rate-limiting step in the conversion of dietary ALA to DHA and EPA. Due to its relatively efficient conversion following consumption, SDA has been targeted as a potential biologically active surrogate for EPA (43). SDA is found in the seeds and leaves of several plants, including those of the boragenase family, such as Echium plantagineum, borage (Boragoofficinalis), evening primose (Oenotherabiennis), and blackcurrent (Ribesnigrum). However, these plants are not adapted to wide scale cultivation, and oil yields are low and variable. That's why the genetic-modified SDA-enriched soybean oil has become the most common source of SDA with a content around 20-30% (44). Very recently, ahiflower oil has been found to be the richest natural source of SDA (20%) in Canada (194). Studies have shown that SDA was able to improve lipid profile (45, 46, 191, 206-208, 215, 229), decrease leukotriene generation (47), inhibit inflammation (48, 220, 230), increase cardiovascular

risk marker omega-3 index (49, 197), attenuate hepatic steatosis (45), reduce atherosclerosis (46) and slow cancer growth (50, 231, 232).

Aβs, consisting of 36-43 amino acids, are derived from the sequential cleavage of the transmembrane amyloid precursor protein (APP) (306). Aβ1-40 is the most abundant form (307). The mechanisms of Aβ-induced neurotoxicity have not been completely understood, but previous studies have demonstrated that exposure of neurons to soluble Aβ could lead to cell apoptosis following oxidative stress and proinflammatory signals (308). Indeed, previous studies showed that the neuronal apoptosis induced by Aβ was evidenced by inhibition of anti-apoptotic Bcl-2 expression and increased expression of pro-apoptotic activated caspase-3, activated caspase-9, Bad, Bax, and Bik (309, 310). It has been demonstrated that Aβ can increase the ROS levels in cultured neurons (311), thereby induce oxidative injury, as evidenced by down-regulating the expression or activity of anti-oxidant enzymes, such as catalase, GSR, and SOD, and up-regulating the expression or activity of pro-oxidant enzymes, such as NOX (312-315). In addition, Aβ has been found to trigger the activation or release of proinflammatory mediators, IL-1β, IL-6, TNFα, MCP-1, COX-2, and TLR4 in neuronal cultures (316-319). Furthermore, it has been demonstrated that Aβ stimulates neurotoxicity through a variety of intracellular signaling pathways, including the activation of JNK, p38, and the inhibition of ERK (311, 317, 319-321). On the other hand, protection of neurons from Aβ-induced toxicity with antioxidants and anti-inflammatory compounds, such as DHA and EPA (322) provides important avenues to ameliorate the pathological effects of Aβ. Treatment of DHA or EPA from fish oil has been demonstrated to exert neuroprotective effects through increasing the expression of Bcl-2 (323, 324) and GPx (325), decreasing the expression of activated caspase-3 (326, 327), activated caspase-9 (328), Bad (165), Bax (323, 324), Bik (165), NAPDH oxidase (329), IL-1β (330), IL-6 (330), TNFα (324, 328),

MCP-1 (331), COX-2 (329), TLR4 (332), and APP (326), improving the activity of catalase (333), up-regulating ERK phosphorylation (334), and down-regulating phosphorylation of JNK (334) and p38 (335). However, the effect of SDA on neurotoxicity is unknown. Therefore, in the present study, we hypothesized that SDA, as a metabolic surrogate for EPA, will suppress apoptosis, oxidative injury, and inflammation induced by Aβ in H19-7 hippocampal cells through the same mechanisms by which DHA and EPA protect against neurotoxicity.

4.3 Materials and methods

4.3.1 Cell culture

The H19-7 cell line was derived from hippocampi dissected from embryonic day 17 (E17) Holtzman rat embryos and immortalized by retroviral transduction of temperature sensitive tsA58 SV40 large T antigen. The cells were generously provided by Dr. Ramesh Jeganathan. All cells were cultured in poly-D-lysine-coated culture dishes and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin-streptomycin (Sigma), 0.001mg/ml puromycin (Sigma), and 0.2 mg/ml G418 (Sigma) in a humidified incubator at 34°C with 5% CO_2.

4.3.2 Fatty acid treatment

Fatty acids (ALA, SDA, DHA and EPA) were purchased from Matreya LLC, (Stage College, PA). Stock solutions of ω-3 fatty acids were in ethanol and further pre-incubated at 34°C for 1 hour in DMEM containing 1.5% of fatty acid-free bovine serum albumin (BSA, Fisher Science) to allow albumin conjugation. After incubation at 34°C for 1 hour, fatty acid-supplemented medium (100 μ M) or BSA-ethanol vehicle control was applied to H19-7 hippocampal cells for two days. Fatty acids were delivered to the cells as fatty acid/BSA complexes.

BSA-ethanol vehicle was used a control. For treatment analysis, cultured H19-7 cells were pre-treated with ω-3 fatty acids followed by exposure to 30 μM Aβ1-40 peptide for 24 h. Aβ1-40 was supplied by Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in deionized distilled water at a concentration of 1 mM and stored at -20°C until use. The stock solutions were diluted to the desired concentrations and pre-incubated at 34°C for 4 days prior to experiments to allow aggregation. After pre-treatment with ω-3 fatty acids, Aβ1-40 in serum-free medium was added to the H19-7 cells. 30 μM of Aβ1-40 peptide was chosen to induce neurotoxicity including oxidative stress, inflammation and apoptosis based on previous studies (336). For Aβ-induced neuroinflammation, 100 ng/ml LPS (Sigma) was added together with Aβ1-40. After treatment, cells were lysed with RIPA lysis buffer.

4.3.3 Fatty acid analysis

H19-7 hippocampal cells incubated with fatty acids for 48 hours were used for fatty acid analysis. Lipid extracts from H19-7 cells were prepared using chloroform/methanol (C/M, 1/1, v/v). The organic phase was collected, dried under N2 gas, and dissolved in C/M 1/1. Saponification and formation of fatty acid methyl esters made from cellular lipid was then performed for liquid chromatography/mass spectrometry (LC/MS). The instrument we used is Agilent 1290 UHPLC coupled Agilent 6460 QQQ triple quadruple mass spectrometer. LC/MS was conducted to quantify the content of DHA and EPA within adipocytes. Palmitic acid-d31 (Sigma, purity > 99%) was added as internal standard. Fatty acid content were normalized to the protein content. Protein quantification was performed using the Bio-Rad DC Protein Assay Kit (Bio-Rad, CA). BSA standard curve and sample preparation and analysis were realized according to manufacturer's instructions.

4.3.4 MTT assay

The MTT staining method was conducted as previously described (337). Briefly, the H19-7 hippocampal cells were seeded in 96-well plates at a density of 2×10^4 cells/ml/well. 100 μM different ω-3 fatty acids were added to the H19-7 cells for 48 or 96 h. For Aβ-induced apoptosis, H19-7 cells were pretreated with 100 μM different ω-3 fatty acids for 48 h and Aβ1-40 was then added to the cells for another 24 h incubation. At the end of the treatment, the culture medium was removed and replaced with sterile-filtered 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT, Sigma Aldrich) solution. After further incubation with MTT solution at 37ºC for 4 hours, the medium was aspirated, allowed to dry completely. Thereafter, 200 μL of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to each well. The microtiter plate was placed on a shaker in order to dissolve the dye. After the formazan crystals had dissolved, the absorbance was determined spectrophotometrically at 490 nm using a reference wavelength of 630 nm on the Bio-Tek spectrophotometer (Winooski, VT).

4.3.5 Total RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

H19-7 cells incubated within different ω-3 fatty acids (100 μM) for 48 h followed by 24-hour induction with Aβ1-40 were washed with PBS and total RNA was extracted using RNeasy Mini Kit (Qiagen; Valencia, CA) according to manufacturer's instructions. The quality and concentration of total RNA was determined spectrophotometrically using NanoDrop (Thermo Scientific). Complementary DNA (cDNA) was synthesized from 1μg of RNA using iScriptTM cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Reverse transcription was performed with sample incubation at 25ºC for 5 minutes, followed by 42ºC for 30 minutes and then 85ºC for 5 minutes. The synthesized cDNA was used immediately for real-time PCR or stored in a -20ºC freezer. Quantitative real-time PCR was performed in the MyiQ single-color real-

time PCR detection thermocycler (Bio-Rad) using iQTM SYBR® Green Supermix (Bio-Rad) to evaluate gene expression. Rat gene specific primers were designed from Primer Bank and constructed by Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA). Oligonucleotide sequences of the primers used for amplification are presented in Table 2. Reaction mixtures were incubated for an initial denaturation at 95ºC for 3 minutes followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 30 seconds, and 55ºC for 10 seconds. The cycle threshold (ΔCT) method was used to measure relative quantification of the target gene, where values were normalized to the reference gene, β-actin. Fold changes of gene expression were calculated by the 2-ΔΔCT method. The statistical analysis was based on ΔCT values.

4.3.6 Western blot analysis

The H19-7 cells were washed with the ice-cold PBS buffer and harvested from the culture plate with cell lysis buffer (RIPA, ThermoFisher Science) containing protease inhibitor cocktail (ThermoFisher Science). The cell lysate was centrifuged at 10,000 g at 4°C for 15 minutes to remove the insoluble material. The protein concentrations were estimated with the Bio-Rad DC Protein Assay Reagent using BSA as a standard. The proteins mixed with sample loading buffer were boiled at 95°C for 5 minutes and then separated in 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. The proteins in the gel were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, CA). The membrane was blocked in 5% non-fat dry milk in the Tris Buffered Saline (TBS) with 0.1% Tween-20. The blocked membrane was incubated with appropriate primary antibodies, and then corresponding secondary antibodies. The membrane was developed using an enhanced chemiluminescent substrate (GE Healthcare, Piscataway, NJ).

4.3.7 Total antioxidant capacity (T-AOC) assay

To measure total antioxidant capacity of H19-7 cells affected by ω-3 fatty acids with Aβ1-40 induction, cells were pretreated with different ω-3 fatty acids (100 μM) for 48 h followed by 24-hour induction with Aβ1-40. After washing with PBS, the total antioxidant potential of samples was determined spectrophotometrically at 570 nm by using a Total antioxidant capacity assay kit (Abcam, Cambridge, UK) according to manufacturer's instructions. This kit measures combined nonenzymatic antioxidant capacity. Briefly, both small molecules and proteins that carry anti-oxidant capacity are able to convert Cu^{2+} ion to Cu^{+} ion. The reduced Cu^{+} ion is chelated with a colorimetric probe that will give a broad absorbance peak around 570 nm, proportional to the total antioxidant capacity. A standard concentration of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used to create a calibration curve and the results of the assay were expressed as nanomoles per microliter Trolox equivalents. Values were normalized to the protein content.

4.3.8 Catalase activity assay

To measure the activity of anti-oxidant enzyme catalase, H19-7 cells treated with different ω-3 fatty acids and induced with Aβ1-40 were collected. After washing with PBS, the catalase enzyme activity of samples was analyzed spectrophotometrically at 570 nm by using a Catalase Assay Kit (Abcam, Cambridge, MA, USA) according to manufacturer's instructions. Briefly, catalase first reacts with H_2O_2 to produce water and oxygen. Thereafter, the unconverted H_2O_2 will react with OxiRed probe to produce a product, which can be measured at 570 nm. A standard concentration of hydrogen peroxide was used to create a calibration curve and the results of the assay were expressed as microunits per microgram protein.

4.3.9 Enzyme-linked immunosorbent assay (ELISA)

The levels of proinflammatory cytokines (IL-1β, IL-6, and TNFα) in the H19-7 hippocampal cells pretreated with different ω-3 fatty acids and induced by Aβ1-40 and LPS were determined with Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, H19-7 cells were pretreated with 100 μM of ALA, DHA, EPA, or SDA for two days, followed by 24-hour incubation with Aβ1-40 and LPS. At the end of the treatment, cell culture supernatant was collected into a centrifuge tube and centrifuged at 10,000 g for 15 minutes at 4℃. The centrifuged supernatant was then ultracentrifuged at 150,000 g for 2 hours at 4°C in a vacuum centrifuge. The ultracentrifuged supernatant samples were immediately stored at -80℃ until use. Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) of the cells was performed for each sample. The supernatant was used in ELISA. The quantity of IL-1β, IL-6, and TNFα in each sample was standardized to its corresponding protein contents.

4.3.10 Statistical analysis

All data are presented as mean ± SEM. The statistical significance of differences between groups was determined by one-way analysis of variance (One-way ANOVA) and Student's t-test (two-tailed). The results were considered to be significant when the value of P was < 0.05. Figures were produced by GraphPad PrismTM version 6.01 (GraphPad software, San Diego, CA).

4.4 Results

4.4.1 SDA effectively converted to EPA in H19-7 cells

As mentioned, SDA bypasses the rate-limiting step in the conversion of dietary ALA to DHA and EPA. Human and animal studies have demonstrated that the consumption of SDA increased EPA levels in many tissues including brain (215, 242). Here, we examined the DHA and

EPA enrichment affected by SDA treatment in vitro in hippocampal cells. To test the conversion of SDA to DHA and EPA, H19-7 cells were incubated in the absence or presence of fatty acids for 48 h. As shown in Figure 6A, cellular content of EPA was increased significantly in hippocampal cells treated with 100 μM of ALA ($p<0.05$), EPA ($p<0.001$), and SDA ($p<0.001$) by 46%, 1188%, and 121%, respectively compared with control. Specifically, SDA increased neuronal EPA content 2.6-fold more efficiently than comparable levels of ALA and was approximately 10% as effective as EPA. While, neuronal DHA content was significantly increased only by 100 μM treatment of DHA ($p<0.001$) (Figure 6B). These results indicate that the protective effects of SDA on Aβ1-40-induced neurotoxicity in H19-7 cells found in the present study may be caused by its downstream metabolite EPA. SDA has little effect on DHA enrichment in H19-7 hippocampal cells. Subsequently, we examined the DHA and EPA enrichment by different concentrations (50, 100, or 200 μM) of SDA. As shown in Figure 6C, treatment of 50, 100, and 200 μM SDA significantly increased the EPA content in H19-7 cells by 65%, 121%, and 96%, respectively. While DHA content was barely affected by SDA treatment (Figure 6D). There results verified that SDA can be a surrogate for EPA, but is not for DHA.

4.4.2 SDA protects H19-7 cells against Aβ-induced cytotoxicity

First, to confirm the safe treatment conditions of ω-3 fatty acids in hippocampal cells, we did the time-response study of 100 μM ω-3 fatty acids on the cell viability using MTT assay. As shown in Figure 7A, cell viability were not statistically changed from the control cells with 100 μM of any ω-3 fatty acid treatment, no matter for 48 h or 96 h. In addition, BSA-ethanol had no effect on H19-7 cell viability. Therefore, 100 μM and 48 h were chosen for ω-3 fatty acid treatment in the present study. Since the cell viability of H19-7 cells was not affected, the improvement of

Aβ-induced H19-7 neurotoxicity by ω-3 fatty acids observed in the following experiments was independent of non-specific toxicity.

To examine whether SDA could protect against Aβ-induced cytotoxicity in H19-7 hippocampal cells, we measured the cell viability using MTT assay after pretreatment of ω-3 fatty acids. As shown in Figure 7B, the cell viability in the 30 μM Aβ1-40-induced cells for 24 h was about 75.0 ± 1.4% of the control values, whereas viability of cells pretreated with DHA, EPA, and SDA at 100 μM for 48 h prior to Aβ1-40 treatment was up to 80.8 ± 1.3%, 81.6 ± 1.3%, and 83.0 ± 1.0%, respectively (Figure 7B). Specifically, SDA improved neuronal viability 1.2-fold and 1.4-fold more efficiently than comparable levels of DHA and EPA. ALA did not restore Aβ-mediated alteration in cytotoxicity (Figure 7B). In conclusion, SDA effectively inhibited Aβ-induced cell death in H19-7 cells and could thereby protect against AD. When compared with other ω-3 fatty acids, the effect of SDA on Aβ-induced neuronal toxicity was the most evident, even better than DHA and EPA.

4.4.3 SDA restored Aβ-induced apoptosis by regulating the expression of apoptotic genes

Since the Bcl-2 family and caspase family are important regulators in various apoptosis pathway, changes in the expression of either the anti-apoptotic or pro-apoptotic family members can affect the execution of apoptosis (338). Previous reports have shown that Aβ strongly decreased anti-apoptotic gene Bcl-2 expression and increased the expression of pro-apoptotic genes, caspase-3, Bad, Bax, and Bik (339, 340). In the present study, to determine whether SDA and other ω-3 fatty acids prevent Aβ-induced apoptosis by modulating the Bcl-2 family and caspase family, mRNA levels of Bcl-2, caspase-3, Bad, Bax, and Bik were observed using quantitative real-time PCR analysis. As shown in Figure 8A, Bcl-2 mRNA expression was markedly decreased after Aβ treatment. Conversely, H19-7 cells pretreated with DHA, EPA, and

SDA prior to Aβ significantly ($P<0.01$) improved the expression of Bcl-2 mRNA by 46%, 25%, and 30%, respectively. Specifically, SDA increased Bcl-2 gene expression 1.2-fold more efficiently than comparable level of DHA and approximately 65% as effective as EPA. ALA did not significantly affect Bcl-2 gene expression. As shown in Figure 8B, caspase-3 mRNA expression was obviously increased after Aβ treatment. On the contrary, H19-7 cells pretreated with DHA and EPA prior to Aβ significantly ($P<0.05$) decreased the expression of caspase-3 mRNA by 51% and 41%, respectively. ALA and SDA did not significantly affect caspase-3 gene expression. As shown in Figure 8C, Bad mRNA expression was clearly increased after Aβ treatment. Inversely, H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ significantly reduced the expression of Bad mRNA by 35% ($P<0.05$), 63% ($P<0.01$), 63% ($P<0.01$), and 36% ($P<0.05$), respectively. Specifically, both ALA and SDA reduced Bad gene expression approximately 56% as effective as DHA or EPA. As shown in Figure 8D, Bax mRNA expression was remarkably increased after Aβ treatment. Conversely, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ significantly inhibited the expression of Bax mRNA by 73% ($P<0.01$), 81% ($P<0.001$), and 59% ($P<0.01$), respectively. Specifically, SDA inhibited Bax gene expression approximately 81% as effective as DHA and approximately 73% as effective as EPA. ALA did not significantly affect Bax gene expression. As shown in Figure 8E, Bik mRNA expression was dramatically increased after Aβ treatment. Inversely, H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ significantly reduced the expression of Bik mRNA by 10% ($P<0.05$), 73% ($P<0.01$), 49% ($P<0.001$), and 51% ($P<0.001$), respectively. Specifically, SDA decreased Bik gene expression 5.1-fold more efficiently than comparable levels of ALA. Meanwhile, SDA reduced Bik gene expression approximately 70% as effective as DHA and almost as the same effective as EPA. Therefore, SDA effectively enhanced the decrease in Bcl-2

expression and attenuated the increase in Bad, Bax, and Bik expression induced by Aβ, and could thereby prevent apoptosis. When compared to other ω-3 fatty acids, the effect of SDA on Aβ-induced expression of Bcl-2 family was much more significant than ALA treatment and very comparable to that of DHA and EPA.

4.4.4 SDA restored Aβ-induced apoptosis by inhibiting the activated caspases expression

Although we found that DHA and EPA significantly decreased the Aβ-induced caspase-3 gene expression, we did not observe a similar effect by SDA. Caspases are initially produced as inactive monomers that require dimerization and often cleavage for activation (341). Once activated, caspases work as cysteine proteases that cleave a critical set of cellular proteins to initiate the apoptotic signal (338). Previous reports showed that the apoptotic cell death induced by soluble Aβ1-40 oligomers required the activation of both caspase-3 and caspase-9 (140, 342). Therefore, we next determined whether SDA and other ω-3 fatty acids prevent Aβ-induced apoptosis by modulating the expression of activated caspase-3 and caspase-9 using Western blot analysis. As shown in Figure 9, the protein levels of activated caspase-3 and caspas-9 in Aβ-treated cells were about 147.4 ± 8.3% and 132.9 ± 0.5% of the values of vehicle-treated cells. However, the protein level of activated caspase-3 in H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ1-40 decreased to 123.7 ± 3.1%, 86.8 ± 3.4%, 78.8 ± 1.3%, and 90.7 ± 2.9%, respectively (Figure 7). Specifically, SDA attenuated the expression of activated caspase-3 2.4-fold more efficiently than comparable levels of ALA and approximately 93% as effective as DHA, 81% as effective as EPA. In addition, the protein level of activated caspase-9 in H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ1-40 reduced to 120.1 ± 2.0%, 68.5 ± 1.6%, 84.8 ± 2.3%, and 105.5 ± 0.8%, respectively (Figure 9). Specifically, SDA attenuated the expression of activated caspase-9 2.1-fold more efficiently than comparable levels of ALA and approximately

44% as effective as DHA, 58% as effective as EPA. Therefore, although SDA may be not as good as DHA and EPA, it effectively inhibited the increase in activated caspase-3 and caspase-9 protein expression in H19-7 cells induced by Aβ1-40 and could thereby prevent apoptosis. When compared to other ω-3 fatty acids, the effect of SDA on Aβ-induced expression of activated caspases was much more significant than ALA treatment and very comparable to that of DHA and EPA.

4.4.5 SDA protects H19-7 cells from Aβ-induced oxidative stress by improving the cellular total anti-oxidative capacity

Evidence indicates that Aβ-induced neuronal cell toxicity is mediated through the excessive oxidative stress (343). Increased intracellular oxidative stress alters multiple signaling pathways that may generate inflammatory response and cell death in neurons, which play key roles in the etiology of neurodegeneration including AD. Oxidative stress occurs due to the impaired anti-oxidant defense systems (344). In the present study, to determine the effects of SDA and other ω-3 fatty acids on the anti-oxidant defense system of Aβ-induced H19-7 cells, intracellular T-AOC was measured using a commercial kit. As shown in Figure 10, T-AOC of the H19-7 cells was markedly decreased after 30 μM Aβ1-40 treatment. Conversely, H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ significantly improved the T-AOC by 2% ($p<0.05$), 19% ($p<0.01$), 21% ($p<0.001$), and 4% ($p<0.01$), respectively (Figure 10). Specifically, SDA increased T-AOC 1.8-fold more efficiently than comparable level of ALA and approximately 22% as effective as DHA, 19% as effective as EPA. To conclude, although not as good as DHA and EPA, 100 μM SDA was sufficient to improve the Aβ-induced decrease in T-AOC of H19-7 cells, and could thereby prevent cell death. In addition, this anti-oxidant activity of SDA was much more significant than that of ALA.

4.4.6 SDA restored the Aβ-induced impairment in total anti-oxidant capacity of H19-7 cells by regulating the expression of anti-oxidant and pro-oxidant genes

Generally, to counteract the increased cellular oxidative stress, the antioxidant enzymes such as catalase, GPx, GSR, and SOD convert the ROS to stable molecules such as water and oxygen. These anti-oxidants are known to protect the cells against oxidative injury (345). On the contrary, the enzyme NOX-1 is one of the major sources of ROS within cells (346). In the present study, to determine whether SDA and other ω-3 fatty acids prevent Aβ-induced oxidative stress by modulating the anti-oxidant and pro-oxidant enzyme gene expression, mRNA levels of catalase, GPx-1, GPx-3, GSR, SOD-1, and NOX-1 were observed using quantitative real-time PCR analysis. As shown in Figure 11A, catalase mRNA expression was not affected after Aβ treatment. Pretreated with DHA, EPA, and SDA prior to Aβ in H19-7 cells seems to increase the expression of catalase, but the data were not significant. As shown in Figure 11B, GPx-1 mRNA expression was obviously decreased after Aβ treatment. Conversely, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ significantly improved the expression of GPx-1 mRNA by 522% ($P<0.001$), 436% ($P<0.05$) and 233% ($P<0.05$), respectively. Specifically, SDA increased GPx-1 gene expression approximately 45% as effective as DHA and 53% as effective as EPA. ALA did not significantly affect GPx-1 gene expression. As shown in Figure 11C, GPx-3 mRNA expression was clearly reduced after Aβ treatment. Inversely, H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ significantly increased the expression of GPx-3 mRNA by 14% ($P<0.05$), 73% ($P<0.01$), 61% ($P<0.01$), and 31% ($P<0.01$), respectively. Specifically, SDA enhanced GPx-3 gene expression 2.2-fold more efficiently than comparable levels of ALA and approximately 42% as effective as DHA, 51% as effective as EPA. As shown in Figure 11D, GSR mRNA expression was remarkably decreased after Aβ treatment. Conversely, H19-7 cells pretreated with DHA, EPA,

and SDA prior to Aβ significantly up-regulated the expression of GSR mRNA by 50% ($P<0.05$), 52% ($P<0.01$), and 21% ($P<0.05$), respectively. Specifically, SDA increased GSR gene expression approximately 41% as effective as DHA or EPA. ALA did not significantly affect GSR gene expression. As shown in Figure 11E, SOD-1 mRNA expression was remarkably down-regulated after Aβ treatment. Inversely, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ significantly improved the expression of SOD-1 mRNA by 102% ($P<0.001$), 152% ($P<0.001$), and 112% ($P<0.01$), respectively. Specifically, the efficacy of SDA in up-regulation of SOD-1 gene expression was almost the same, and even a little bit better than that of DHA. The efficacy of SDA in up-regulation of SOD-1 gene expression was approximately 74% as effective as EPA. ALA did not significantly affect SOD-1 gene expression. As shown in Figure 11F, NOX-1 mRNA expression was dramatically increased after Aβ treatment. Conversely, H19-7 cells pretreated with DHA prior to Aβ significantly reduced the expression of NOX-1 mRNA by 48% ($P<0.01$). Pretreated with ALA, EPA, and SDA prior to Aβ in H19-7 cells seems to decrease the expression of NOX-1, but the data were not significant. Therefore, SDA effectively enhanced the decrease in the gene expression of catalase, GPx-1, GPx-3, GSR, SOD-1 induced by Aβ, and could thereby prevent oxidative stress. When compared to other ω-3 fatty acids, the effect of SDA on above Aβ-induced oxidative injury was much more significant than ALA treatment and very comparable to that of DHA and EPA.

4.4.7 SDA restored the Aβ-induced impairment in total anti-oxidant capacity of H19-7 cells by improving the anti-oxidant catalase activity

Since we haven't found any change in catalase gene expression after Aβ1-40 treatment, catalase enzymatic activity was next studied. As shown in Figure 12, catalase activity of the H19-7 cells was not significantly changed after 30 μM Aβ1-40 treatment. However, H19-7 cells

pretreated with ALA, DHA, EPA, and SDA prior to Aβ all significantly improved the catalase activity by 12% ($p<0.05$), 36% ($p<0.001$), 43% ($p<0.001$), and 23% ($p<0.01$), respectively when compared with the control BSA-ethanol vehicle cells (Figure 12). Specifically, SDA increased catalase activity 1.9-fold more efficiently than comparable level of ALA and approximately 64% as effective as DHA, 53% as effective as EPA. To conclude, ω-3 fatty acids are naturally anti-oxidants that can improve the activity of the anti-oxidant enzyme catalase in neurons. The anti-oxidant properties of ω-3 fatty acids allows them to also have neuroprotective ability.

4.4.8 SDA protects H19-7 cells from Aβ+LPS-induced release of proinflammatory cytokines by regulating the expression of related genes

Considering that the neuroinflammation response plays a significant role in modulating AD progression (347) and Aβ was reported to induce neuronal inflammation in brain tissues including hippocampus (317, 319-321), we further examined whether SDA and other ω-3 fatty acids had anti-inflammatory action on the Aβ-stimulated cells. The levels of inflammatory cytokines including IL-1β, IL-6, and TNFα were evaluated by ELISA analysis. However, treatment of Aβ1-40 alone in H19-7 cells for 24 h was not able to induce the release of these proinflammatory cytokines (data not shown). LPS was reported to bind with the TLR4 receptors on the neuronal membrane to stimulate the production of pro-inflammatory cytokines (348, 349). Therefore, 100 ng/ml LPS was added together with Aβ1-40 for 24 h after pretreatment of ω-3 fatty acids. As shown in Figure 13A, B and C, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant increase in the release of IL-1β ($p<0.05$), IL-6 ($p<0.01$), and TNFα ($p<0.01$). These proinflammatory cytokines were significantly inhibited by ω-3 fatty acids. As shown in Figure 13A, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ+LPS significantly ($p<0.05$) reduced the IL-1β release by 25%, 27%, and 18%, respectively when

compared with the Aβ+LPS-treated cells. Specifically, SDA decreased the IL-1 β release approximately 72% as effective as DHA and approximately 67% as effective as EPA. ALA did not significantly affect Aβ+LPS-induced IL-1β production. As shown in Figure 13B, H19-7 cells pretreated with DHA prior to Aβ+LPS significantly ($p<0.05$) decreased the release of IL-6 by 38% when compared with the Aβ+LPS-treated cells. However, pretreatment with ALA, EPA, and SDA did not significantly affect IL-6 production. As shown in Figure 13C, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ+LPS significantly inhibited the production of TNFα by 62% ($p<0.01$), 46% ($p<0.05$), and 34% ($p<0.05$), respectively when compared with the Aβ+LPS-treated cells. Specifically, SDA decreased the TNFα level approximately 55% as effective as DHA and approximately 74% as effective as EPA. ALA did not significantly affect Aβ+LPS-induced TNFα production. To summarize, SDA effectively inhibited the release of pro-inflammatory cytokines IL-1β and TNFα triggered by Aβ+LPS, and could thereby prevent neuroinflammation. When compared to other ω-3 fatty acids, the effect of SDA on Aβ+LPS-induced neuroinflammation was much more significant than ALA treatment and very comparable to that of DHA and EPA.

Correspondingly, we next measured the mRNA levels of the above proinflammatory cytokines using qRT-PCR analysis. As shown in Figure14A, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant increase in the gene expression of IL-1β to 6.5-fold of the untreated control. H19-7 cells pretreated with DHA and EPA prior to Aβ+LPS significantly ($p<0.05$) decreased the mRNA level of IL-1β to 2.3 and 2.2-fold of the control group. Pretreatment with ALA and SDA did not significantly affect IL-1β gene expression, though there was a trend to decrease. As shown in Figure14B, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant increase in the gene expression of IL-6 to 3.9-fold of the untreated control. Pretreatment with ALA, DHA, EPA, and SDA all did not

significantly affect IL-6 gene expression. As shown in Figure14C, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant increase in the gene expression of TNFα to 3.0-fold of the untreated control. H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ+LPS significantly ($p<0.05$) down-regulated the mRNA level of TNFα to 1.7, 2.1, and 1.9-fold of the control group. Specifically, SDA reduced the TNFα gene expression approximately 84% as effective as DHA and 1.2-fold more efficiently than comparable levels of EPA. Pretreatment with ALA did not significantly affect TNF α gene expression. In conclusion, consistent with the ELISA results, SDA had no significant effect on the Aβ-induced gene expression of IL-6 and IL-1β, though with trends to decrease. In addition, SDA pretreatment significantly reduced the Aβ-induced TNFα release as well as down-regulated its gene expression.

Besides these proinflammatory cytokines, we also tested the gene expression of some other inflammatory mediators. COX-2 plays crucial role in mediating inflammatory response, which promotes the formation of prostanoids. Previous reports have shown that Aβ strongly increased COX-2 expression in cells (350). As shown in Figure14D, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant increase in the gene expression of COX-2 to 24.9-fold of the untreated control. H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ+LPS significantly ($p<0.001$) inhibited the mRNA level of COX-2 to 2.0, 3.8, and 6.1-fold of the control group. Specifically, SDA decreased the COX-2 gene expression approximately 82% as effective as DHA and approximately 88% as effective as EPA. Pretreatment with ALA did not significantly affect COX-2 gene expression. MCP-1 plays a role in the recruitment of circulating monocytes to sites of inflammation. Evidence has implicated that Aβ deposition can trigger the expression of MCP-1 in the brain of AD patients (351). As shown in Figure14E, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant

increase in the gene expression of MCP-1 to 7.6-fold of the untreated control. H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ+LPS significantly down-regulated the mRNA level of MCP-1 to 3.9 ($p<0.001$), 4.4 ($p<0.01$), and 5.7-fold ($p<0.01$) of the control group. Specifically, SDA reduced the MCP-1 gene expression approximately 52% as effective as DHA and approximately 60% as effective as EPA. Pretreatment with ALA did not significantly affect MCP-1 gene expression. TLR4 plays a key role in the recognition of LPS and initiation of inflammatory response. Previous studies reported that TLR4 expression increased in neurons when exposed to Aβ (352). As shown in Figure14F, the exposure of H19-7 cells to 30 μM Aβ1-40 and 100 ng/ml LPS for 24 h resulted in a significant increase in the gene expression of TLR4 to 11.5-fold of the untreated control. H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ+LPS significantly reduced the mRNA level of TLR4 to 4.1 ($p<0.01$), 7.1 ($p<0.05$), and 8.2-fold ($p<0.05$) of the control group. Specifically, SDA inhibited the TLR4 gene expression approximately 45% as effective as DHA and approximately 76% as effective as EPA. Pretreatment with ALA did not significantly affect TLR4 gene expression. Therefore, SDA effectively inhibited the mRNA levels of pro-inflammatory mediators COX-2, MCP-1, and TLR4 triggered by Aβ+LPS, and could thereby prevent neuroinflammation. When compared to other ω-3 fatty acids, the effect of SDA on Aβ+LPS-induced expression of pro-inflammatory mediators was much more significant than ALA treatment and very comparable to that of DHA and EPA.

4.4.9 SDA protects H19-7 cells from Aβ-induced gene expression of APP

APP is the precursor molecule whose proteolysis generates Aβ. Accumulation of APP has been implicated in the induction of oxidative stress-mediated neural death by disrupting mitochondrial homeostasis (353, 354). Previous reports have shown that Aβ strongly increased APP gene expression in neurons (355). In the present study, to determine whether SDA and other

ω-3 fatty acids prevent Aβ-induced neurotoxicity by affecting the expression of APP, the mRNA level of APP was measured using qRT-PCR analysis. As shown in Figure 15, the exposure of H19-7 cells to 30 μM Aβ1-40 for 24 h resulted in a significant increase in the gene expression of APP to 82.4-fold of the untreated control. H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ1-40 significantly down-regulated the mRNA level of APP to 58.2 ($p<0.001$), 4.7 ($p<0.001$), 21.2 ($p<0.001$), and 47.7-fold ($p<0.05$) of the control group (Figure 15). Specifically, SDA reduced the APP gene expression 1.4-fold more efficiently than comparable levels of EPA and approximately 45% as effective as DHA, 57% as effective as EPA. Therefore, SDA effectively inhibited the mRNA level of APP induced by Aβ, and could thereby prevent neurotoxicity. When compared to other ω-3 fatty acids, the effect of SDA on Aβ-induced APP expression was much more significant than ALA treatment and very comparable to that of DHA and EPA.

4.4.10 SDA protects H19-7 cells from Aβ-induced neurotoxicity by regulating the phosphorylation of MARKs

MAPKs signaling pathways are involved in Aβ-induced neurotoxicity. MAPKs include three subtypes: JNK, p38, and ERK, whom are associated with oxidative stress and proinflammatory response induced cell apoptosis signaling, thereby play important roles in pathological processes of AD (356, 357) Phosphorylation of JNK and p38 is associated with cytotoxicity, while phosphorylation of ERK promotes cell survival and proliferation (358). Aβ induces neurotoxicity through an apoptotic event mediated partially by the generation of oxidative stress, which can further induce expression of proinflammatory cytokines via activation of JNK, p38, and ERK pathways (359). Previous reports have shown that Aβ strongly stimulated the activation of JNK and p38, while inhibited the phosphorylation of ERK (140, 317, 320). In the present study, to determine whether SDA and other ω-3 fatty acids prevent Aβ-induced

neurotoxicity by modulating the phosphorylation of MARKs, the protein levels of phosphorylated-JNK, phosphorylated-p38, and phosphorylated-ERK was measured using Western blot analysis. As shown in Figure 16A, the exposure of H19-7 cells to 30 μM Aβ1-40 for 24 h resulted in a significant phosphorylation of JNK ($p<0.001$). Conversely, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ significantly reduced the activation of JNK by 20%, 41%, and 15%, respectively when compared with the Aβ-treated cells. Specifically, SDA decreased JNK phosphorylation approximately 75% as effective as DHA and approximately 37% as effective as EPA. ALA did not significantly affect Aβ-induced JNK activation. As shown in Figure 16B, the exposure of H19-7 cells to 30 μM Aβ1-40 for 24 h resulted in a significant phosphorylation of p38 ($p<0.001$). On the contrary, H19-7 cells pretreated with DHA, EPA, and SDA prior to Aβ significantly reduced the activation of p38 by 27%, 16%, and 14%, respectively when compared with the Aβ-treated cells. Specifically, SDA decreased p38 phosphorylation approximately 52% as effective as DHA and approximately 88% as effective as EPA. ALA did not significantly affect Aβ-induced p38 activation. As shown in Figure 16C, the exposure of H19-7 cells to 30 μM Aβ1-40 for 24 h resulted in a significant inhibition of ERK phosphorylation ($p<0.001$). Inversely, H19-7 cells pretreated with ALA, DHA, EPA, and SDA prior to Aβ significantly improved the activation of ERK by 58%, 217%, 72%, and 185%, respectively when compared with the Aβ-treated cells. Specifically, SDA increased ERK phosphorylation 3.2-fold more efficiently than comparable levels of ALA, 2.6-fold more efficiently than comparable levels of EPA, and approximately 85% as effective as DHA. Therefore, SDA effectively protected the H19-7 cells from the Aβ-induced JNK and p38 apoptotic signaling and improved the cellular ERK survival signaling, could thereby prevent neurotoxicity. When compared to other ω-3 fatty acids, the effect

of SDA was much more significant than ALA treatment and very comparable to or even better than that of DHA and EPA.

4.5 Discussion

Accumulating evidence supports the notion that the incidence and prevalence of AD are significantly influenced by nutritional factors. Adequate dietary availability of DHA and EPA is fundamental to brain function. DHA and EPA have been found to protect against Aβ-induced apoptosis, oxidative injury, and inflammation in neurons through regulation of MAPKs signaling (146, 287). SDA has been targeted as a potential biologically active surrogate for EPA since SDA can be effectively converted to EPA by the body and shares many beneficial properties with EPA (360). In the present study, we examined and compared the protective effects of these fatty acids on Aβ-induced stress-inflammatory-apoptotic signaling in H19-7 hippocampal cells. Since ALA is the precursor of ω-3 fatty acids in the metabolic pathway and can also be converted to EPA, we also compared the influence of SDA with ALA on Aβ-induced neurotoxicity in our experiment. The effects of DHA and EPA directly were also investigated. We demonstrated that 100 μM is a safe concentration for ω-3 fatty acid treatment by MTT assay. Therefore, the neuroprotective effects of SDA and other investigated ω-3 fatty acids found in this study was independent of non-specific toxicity.

SDA is the Δ6-desaturase product of ALA in the bioconversion of ALA to EPA. In humans, the conversion of ALA to EPA is in low amounts (less than 7%) and in even low amounts to DHA (less than 1%) due to the rate-limiting enzyme (250). Therefore, nutritional supplementation with ALA was not sufficient to induce long chain ω-3 PUFA accumulation (251). In the present study, we hypothesized that by skipping the rate-limiting step, the conversion of SDA to EPA would be

more efficient than that of ALA to EPA. By LC/MS analysis of DHA and EPA enrichment, we demonstrated that SDA increased EPA content in H19-7 cells by 121% compared to control. The efficacy of EPA enrichment by SDA was about 2.6-fold greater than comparable levels of ALA and is about 10% of the enrichment by EPA. On the other hand, both ALA and SDA, even EPA was not able to increase DHA content effectively in H19-7 cells. This is probably due to another rate limiting step converting docosapentaenoic acid (DPA) to DHA (42). These findings agree with those of previous studies. Studies have shown that consumption of SDA as ethyl esters, echium oil, or SDA-soybean oil increased EPA levels in red blood cells (49, 197, 242, 252-255), peripheral blood mononuclear cell (254), plasma (47, 242, 256), and neutrophils (256). Feeding with SDA increased the EPA content in many tissues of rodents including brain (215). The efficacy of SDA on EPA enrichment in different tissues ranges from 17% to 85% as much as the efficacy of EPA on EPA enrichment based on human studies (49, 197, 242, 253-255). Furthermore, the efficacy of SDA was found to be 1.9 to 4.3-fold as much as that of ALA on EPA enrichment in human studies (242, 252). Nearly all studies found no significant change in DHA content after SDA supplementation. Based on these findings, we may conclude that SDA consumption would be expected to confer the health benefits associated with consumption of EPA, but not DHA.

Hippocampal formation is important for memory (361). In rat, the performance of spatial memory has been found to be tightly associated with hippocampal activity (362). The apoptosis of hippocampal neurons played a key role in the learning and memory deficit (363). At early stages of AD, hippocampus is especially vulnerable to damage (364). H19-7 cell line, which derived from the embryonic rat hippocampus, is known to possess the characteristics of hippocampal neurons. Under microscope, H19-7 cells express morphological markers of neuronal, glial, or bipotential lineage. H19-7 cell line is widely used in studies of AD (284, 315). Aβ1-40 has been found to

induce neurotoxicity in H19-7 cells (315). Therefore, H19-7 hippocampal cell line was used in the present study.

A large body of evidence has shown that Aβ-induced apoptotic neuronal death occurs through the activation of apoptotic pathways (365). Apoptosis is a tightly regulated process, which involves changes in the expression of a distinct set of genes, such as Bcl-2 family members and caspases (338). Aβ disrupts the function of mitochondria, which is the central to the oxidative stress regulation in AD (366). Bcl-2, as a mitochondrial membrane-associated protein, subsequently exerts its anti-apoptotic effect by inhibiting the expression of pro-apoptotic Bad, Bax, Bik, and thereby reducing the activation of caspase-3 and caspase-9 (367). In the present study, Aβ-induced cytotoxicity was assessed by MTT assay. Aβ1-40 treatment at 30 μM for 24 h significantly decreased cell viability. However, pretreatment with SDA, similar to DHA and EPA, markedly attenuated the loss of cell viability by down-regulating the expression of Bad, Bax, and Bik, up-regulating Bcl-2 expression, and inhibiting the activation of caspase-3 and caspase-9. The results are consistent with previous studies. It has been showed that fish oil administration decreased the expression of pro-apoptotic Bax and caspase-3, thereby rescued hippocampal neurons from apoptosis (368). Many other substances that have anti-apoptotic effects in Aβ-induced neurons were found to also act through elevated Bcl-2, decreased Bax, and inhibited caspase activation in hippocampus (311, 369). Since mitochondria dysfunction is the initiation of Aβ-induced apoptosis; DHA and EPA were demonstrated to improve mitochondria function in neurons (290, 370), future studies should be conducted to examine the protective effect of SDA on mitochondrial function. In addition, MTT assay cannot distinguish apoptotic death from all cell death, so measurement of specific apoptotic cell death, such as TUNEL assay should be conducted in future studies. In summary, these data indicated that although not as significant as DHA and

EPA, treatment of SDA effectively exerted its neuroprotective property via inhibition of the apoptotic pathway.

Increased oxidative injury has been reported in the early stages of mild cognitive impairment in the brains of AD patients (371). As natural antioxidants, DHA and EPA have been reported to prevent oxidative stress-mediated apoptosis in cultured neurons associated with AD (32, 140). It is well established that Aβ is neurotoxic via an oxidative stress-dependent apoptotic process. It has been demonstrated that intracerebroventricular administration of Aβ1-40 resulted in dramatic decrease in T-AOC level in rats (372). We also observed that Aβ1-40 disrupted T-AOC level in H19-7 cells. One possible approach to prevent oxidative stress-mediated cellular injury is to augment oxidative defense capacity through dietary intake of antioxidants. DHA and EPA have been found to significantly improve the T-AOC decline in neurons or in RPE cells (32, 373). One of the purposes of the current study was to evaluate whether ω-3 fatty acids can successfully inhibit the Aβ-induced oxidative injury in the rat hippocampal cells (H19-7). T-AOC is an indicator of total intracellular antioxidant status, which activity reflects cell damage and can be used to determine the degree of cell damage. In the present study, pretreatment with ω-3 fatty acids, DHA, EPA, and SDA could significantly increase the T-AOC activity in Aβ-induced H19-7 cells, suggesting that the protective effects of them might be partly associated with its anti-oxidative function. It has been reported that Aβ produces H_2O_2, leading to hydroxyl radical formation via the Fenton reaction in the presence of transition metal ions (374). In the present study, we observed that Aβ increased the expression of NOX, and decreased the expression of GPx, GSR, and SOD in H19-7 cells. Induction of endogenous anti-oxidative enzymes appears to be the first line of defense in the brain to counteract the toxic effects of oxidative stress associated with AD (375). The protective properties of antioxidants are partially attributed to their ability to

induce antioxidant enzymes. Because ω-3 fatty acids have strong anti-oxidative properties, it was hypothesized in the present study that the enhancement of Aβ-induced T-AOC disruption by ω-3 fatty acids may be due to inhibition of pro-oxidative NOX expression and increase of anti-oxidative enzyme expression, such as catalase, GSR, GPx, and SOD. NOX transfers electrons from NADPH inside the cell across the membrane and coupling these to molecular O_2 to produce reactive free-radical superoxide (376). Catalase and GPx catalyze the decomposition of H_2O_2 or other peroxides in cell membranes and then converts H_2O_2 into O_2, and H_2O (377, 378). GSR catalyzes the reduction of pro-oxidative glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), resisting oxidative stress and maintaining the reducing environment of the cell (379). SOD specifically catalyzes the dismutation of O_2^- to O_2 and H_2O_2 (380). Treatment of DHA or EPA has been found to restore the increased expression of NOX, the decreased expression or activity of catalase, GPx, GSR, and SOD in brains associated with AD (329, 381-384). In the present study, NOX-1 expression induced by Aβ was significantly reduced by DHA. Pretreatment of EPA and SDA did not significantly affect NOX-1 expression. This is consistent with our results that SDA could effectively concert to EPA, but not DHA in H19-7 cells and suggesting that DHA may have its unique mechanism in mediating neuroprotective effects. Catalase expression was not significantly affected by any ω-3 fatty acid treatment, but the activity of catalase was significantly improved by pretreatment of DHA, EPA, and SDA. The expression of GPx-1, GPx-3, GSR, and SOD-1 depressed by Aβ was all significantly increased by pretreatment of DHA, EPA, and SDA. These results are consistent with previous studies and it is the first time that we report SDA act as a cytoprotective agent against Aβ-induced nerutoxicity.

Activation of neuroinflammation has been regarded as one of the underlying mechanism of AD pathology. Studies have shown that Aβ deposit contributes to the neuroinflammation

associated with AD (385). Neurons can produce pro-inflammatory mediators, such as IL-1β, IL-6, TNFα, COX-2, MCP-1, and TLR4 (317, 318, 386, 387). H19-7 hippocampal cells expressed all three IL-1 receptor, IL-6 receptor, and TNF receptor, which were functional upon stimulation (284, 388). It has been demonstrated that Aβ stimulated the release of IL-1β, IL-6, and TNFα from organotypic hippocampal culture (320). A β treatment could also increase levels of pro-inflammatory mediators, such as COX-2, MCP-1, and TLR4 in neurons (317, 318, 389). Consistently, neurons in the AD brains display increased levels of IL-1β, IL-6, TNFα, COX-2, and MCP-1 (390). However, treatment with 30 μM Aβ for 24 h was not sufficient to induce significant release of proinflammatory cytokines in H19-7 cells. Compared with microglial and astrocytes, which are recognized as the macrophages in brain, neurons like H19-7 cells produce much less proinflammatory cytokines, although H19-7 cells has glial morphology and property (391). Therefore, LPS were added together with Aβ to induce neuroinflammation. In fact, LPS is widely used to induce neuroinflammation (392). In the present study, we found that DHA, EPA, and SDA all could reverse the Aβ and LPS-induced release of IL-1β and TNFα; DHA could reverse the release of IL-6. Consistent with the ELISA result, DHA, EPA, and SDA significantly down-regulated the Aβ and LPS-induced TNFα expression; DHA and EPA significantly reduced the IL-1β expression; however, no effect has been found by any investigated fatty acid on IL-6 gene expression. These results are consistent with previous studies, which have shown that SDA was able to decrease leukotriene generation (47) and inhibit inflammation (48). IL-1β has been demonstrated to stimulate IL-6 production in H19-7 cells (284). In contrast, IL-1 receptor immunoblockade reduced IL-6 production by hippocampal cultures (393). Therefore, the release of IL-1β induced by Aβ and LPS found in the present study might further increase IL-6 production, thereby exacerbate neuroinflammation. COX-2 is an enzyme which catalyzes the formation of

prostanoids, and promotes the expression of IL-1β, IL-6, and TNFα (394). MCP-1 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation upon stimuli (395). TLR4 recognizes many pathogen-related molecules and endogenous proteins, such as Aβ and LPS, then activating immune signaling and promoting release of proinflammatory cytokines (396). In the present study, we found that DHA, EPA, and SDA all could reverse the Aβ-induced increased in COX-2, MCP-1, and TLR4 expression in H19-7 cells. These findings suggest that COX-2 and TLR4 may be potential targets of Aβ, and COX-2 inhibitors and TLR4 blockers might be target for attenuating Aβ-induced neuronal toxic associated with AD.

Lipid homeostasis plays an important role in the maintenance of a normal brain function (397). Lipid composition is determinant for the biophysical properties of neuronal membranes (342). Lipid rafts, which locate across neuronal cell membranes, serve as regulators of membrane fluidity, organizing centers for the assembly of signaling molecules, and thereby mediate membrane receptor trafficking (398). Lipid raft disorganization is characterized by loss of cholesterol and subsequent selective protein displacement, which is associated with a reduced activity of plasmin, a serine protease able to degrade Aβ peptide in AD brains (399). PUFA enrichment impact on lipid rafts accompanied by local lipid redistribution, leading to improvement of membrane viscosity and modulation of cellular apoptosis and survival signaling pathways. (400, 401). The mechanism underlying Aβ-induced neurotoxicity is complex, involving several signaling pathways. Recent evidence suggests that Aβ could stimulate JNK and p38 activation, which might be involved in AD pathogenesis (311, 402-404). It remains controversial for Aβ-induced ERK signaling; some studies showed activation of ERK phosphorylation (321, 402, 403), some demonstrated inhibition of ERK phosphorylation (140), while some found stable phosphorylation profile of ERK after Aβ treatment (311). Here, we found that phosphorylated

JNK and p38 were markedly increased, while phosphorylated ERK was dramatically decreased after Aβ1-40 treatment. Generally, phosphorylation of JNK and p38 is highly activated in response to a variety of stress signals, including oxidative stress and proinflammatory cytokines, while the activation of ERK pathway promotes cell growth, differentiation and survival (358). Previous studies demonstrated that the neuroprotective effect of DHA and EPA are mediated through JNK, p38, and ERK pathways. JNK inhibitor, SP600125 was found to significantly block the neuroprotective effects by DHA (36) and EPA (146) in cultured neurons. DHA has been shown to inhibit the synthesis of inflammatory products in activated microglia by inhibition of p38 phosphorylation (405). Furthermore, DHA pre-treatment was demonstrated to significantly increase neuronal survival upon Aβ treatment by promoting ERK-related survival pathway (140). ERK inhibitor, U0126, abolished DHA-induced ERK phosphorylation and neurogenesis in human neuronal cells (406). Here, we found that DHA, EPA, and SDA were all able to block the activation of JNK/p38 phosphorylation induced by Aβ and meanwhile improve ERK phosphorylation depressed by Aβ. ERK activity is mediated by Ras, but the activities of JNK, and p38 are Ras-independent (407), suggesting that the effects of fatty acids are associated with both Ras-dependent and Ras-independent pathways. Among the investigated ω-3 fatty acids, DHA was shown to be the most effective in neuroprotection induced by Aβ. This might be explained by the fact that DHA is the most abundant PUFA in neuronal phospholipids (408) and DHA is the precursor of neuroprotection D1 (409). Besides the effect on lipid rafts, EPA specifically had an influence on eicosanoid biosynthesis. In cell membrane, EPA competes with ω-6 PUFA AA, resulting in reduced levels of pro-inflammatory metabolites of AA, and increased levels of anti-inflammatory metabolites of EPA (275). In the present study, we demonstrated the similar effects by SDA, which exerted its neuroprotective properties against Aβ by inhibiting stress-JNK/p38 signaling and

improving ERK-survival signaling. Future experiments are suggested to identify the upstream anti-apoptotic pathways triggered by ω-3 fatty acids in neurons.

4.6 Conclusion

In summary, our results suggest that the ω-3 fatty acid, SDA provides hippocampal neurons with a higher resistance level to the cytotoxic effects induced by Aβ through (1) effectively converting to neuroprotector EPA, (2) regulating expression of apoptotic mediators, (3) improving total anti-oxidant capacity by increasing expression or activity of anti-oxidant enzymes, (4) reducing expression of pro-inflammatory mediators, (5) inhibiting expression of the precursor of Aβ, (6) attenuating stress-triggered apoptotic JNK/p38 phosphorylation, and (7) activating survival-related ERK signaling pathway. This is the first time that such protective properties have been reported with SDA. A diet rich in ω-3 fatty acids may therefore reduce Aβ-mediated cytotoxicity, neuronal loss and the risk of developing AD.

Table 2 Oligonucleotide primer sequences used in real-time PCR.

Gene	Forward primer	Reverse primer
APP	5’-TCAGATTGCGATGTTCTGTGG-3’	5’-CTGGCTGGTTTGCTTCCATCA-3’
Bad	5’-AAGTCCGATCCCGGAATCC-3’	5’-GCTCACTCGGCTCAAACTCT-3’
Bax	5’-TGAAGACAGGGGCCTTTTTG-3’	5’-AATTCGCCGGAGACACTCG-3’
Bcl-2	5’-GTCGCTACCGTCGTGACTTC-3’	5’-CAGACATGCACCTACCCAGC-3’
Bik	5’-ACTGTTCCACACGACCAGG-3’	5’-CACAGGACTAAGGTTTTCCCC-3’
Caspase-3	5’-ATGGAGAACAACAAAACCTCAGT-3’	5’-TTGCTCCCATGTATGGTCTTTAC-3’
Catalase	5’-AGCGACCAGATGAAGCAGTG-3’	5’-TCCGCTCTCTGTCAAAGTGTG-3’
COX-2	5’-TGAGCAACTATTCCAAACCAGC-3’	5’-GCACGTAGTCTTCGATCACTATC-3’
GPx-1	5’-AGTCCACCGTGTATGCCTTCT-3’	5’-GAGACGCGACATTCTCAATGA-3’
GPx-3	5’-TCACACTTTCTCCAGGTTCCCGTT-3’	5’-TCATGTGGGCATATGGGAGATGCT-3’
GSR	5’-GACACCTCTTCCTTCGACTACC-3’	5’-CCCAGCTTGTGACTCTCCAC-3’
IL-1β	5’-GCAACTGTTCCTGAACTCAACT-3’	5’-ATCTTTTGGGGTCCGTCAACT-3’
IL-6	5’-TAGTCCTTCCTACCCCAATTTCC-3’	5’-TTGGTCCTTAGCCACTCCTTC-3’
MCP-1	5’-TTAAAAACCTGGATCGGAACCAA-3’	5’-GCATTAGCTTCAGATTTACGGGT-3’
NOX-1	5’-GGTTGGGGCTGAACATTTTTC-3’	5’-TCGACACACAGGAATCAGGAT-3’
SOD-1	5’-AACCAGTTGTGTTGTCAGGAC-3’	5’-CCACCATGTTTCTTAGAGTGAGG-3’
TLR4	5’-GCCTTTCAGGGAATTAAGCTCC-3’	5’-AGATCAACCGATGGACGTGTAA-3’
TNFα	5’-CCCTCACACTCAGATCATCTTCT-3’	5’-GCTACGACGTGGGCTACAG-3’
β-actin	5’-GGCTGTATTCCCCTCCATCG -3’	5’-CCAGTTGGTAACAATGCCATGT-3’

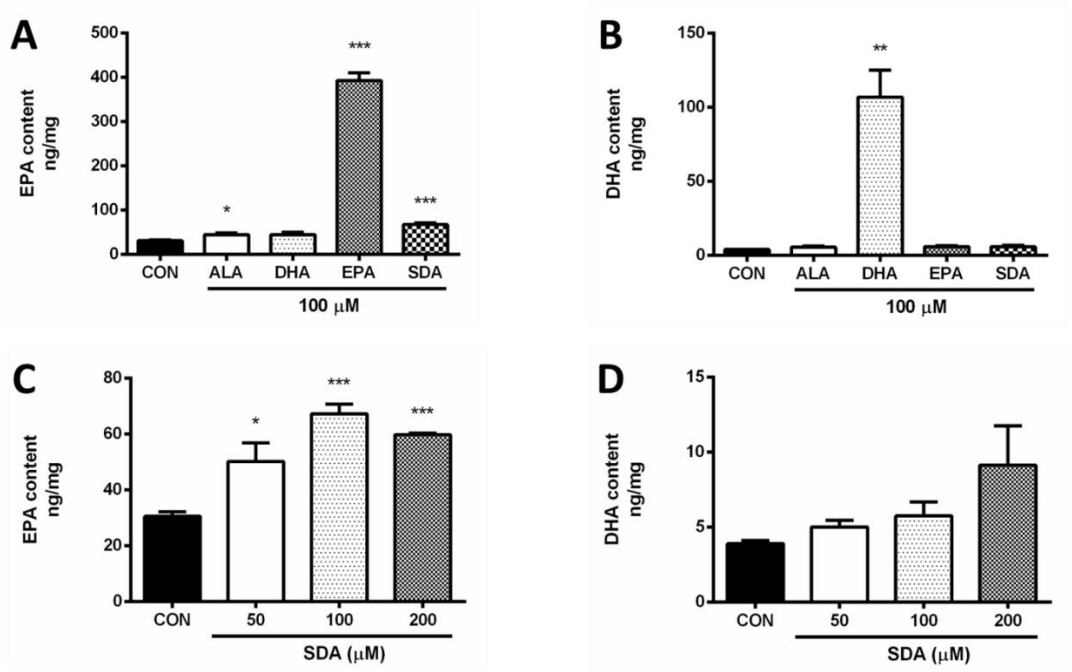

Figure 6 Enrichment of DHA and EPA by ω-3 fatty acid treatment in H19-7 cells.

H19-7 hippocampal cells were treated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. EPA **(A, C)** and DHA **(B, D)** content was measured by LC/MS. Values were obtained from three independent experiments and were expressed as the means ± SEM. Data were normalized to the protein contents; *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

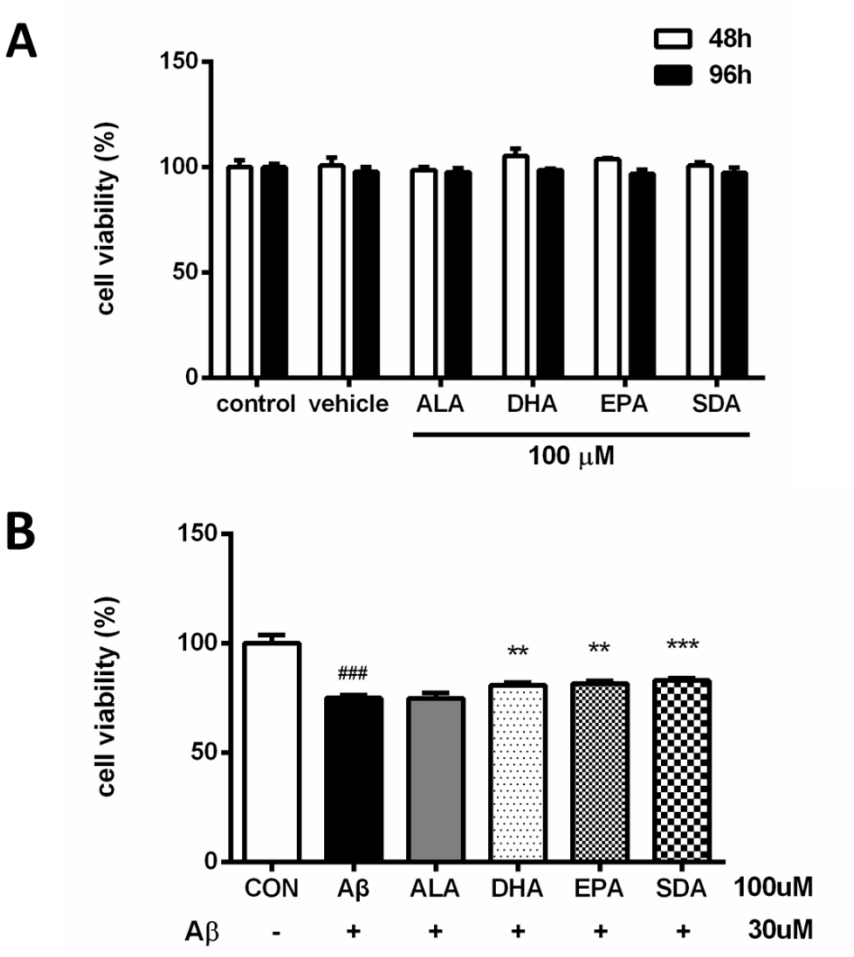

Figure 7(A) Effect of different ω-3 fatty acids (100 μM) on cell viability of H19-7 cells. (B) Pre-incubation with DHA, EPA, and SDA protects H19-7 cells from soluble Aβ neurotoxicity.

(A) H19-7 cells were treated with ALA, DHA, EPA, SDA, or BSA-ethanol (vehicle) in the concentration of 100 μM for 48 h or 96 h. (B) H19-7 cells were cultured for 48 h in medium enriched with different *ω*-3 fatty acids prior to exposure to non-fibrillar Aβ for 24 h. Cell viability was then monitored using the MTT assay. Values were obtained from n=4-8 independent experiments and were expressed as the means ± SEM; Data were normalized to the effect of control designated as 100%; $^{###}P < 0.001$, different from BSA-ethanol vehicle treated control cells; $^{**}P < 0.01$, $^{***}P < 0.001$, different from Aβ1-40-treated cells.

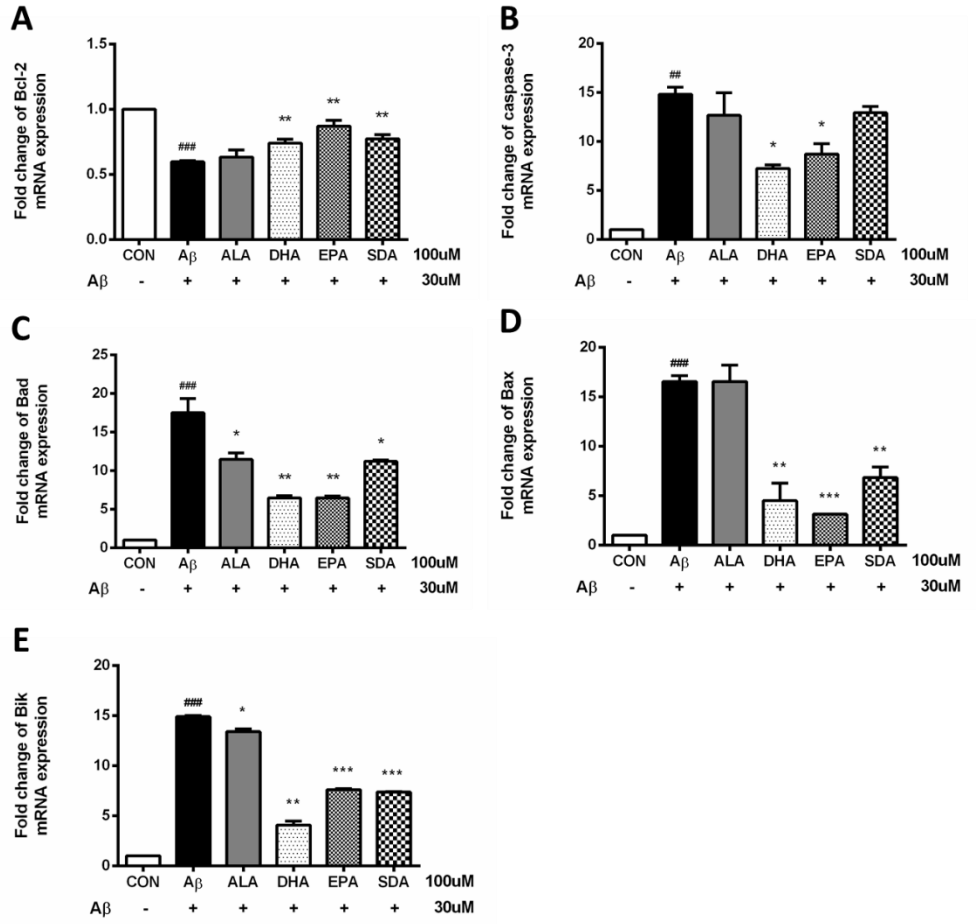

Figure 8 Effects of different ω-3 fatty acids on the apoptotic gene expression in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. The mRNA expression of anti-apoptotic gene Bcl-2 (A) and pro-apoptotic gene caspase-3 (B), Bad (C), Bax (D), and Bik (E) in H19-7 cells were analyzed by quantitative real-time PCR. Values were obtained from three independent experiments and were expressed as the means ± SEM; Data were normalized to the level of β-actin mRNA. $^{\#\#}P<0.01$, $^{\#\#\#}P < 0.001$, different from BSA-ethanol vehicle treated control cells; $^{*}P<0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from Aβ1-40-treated cells.

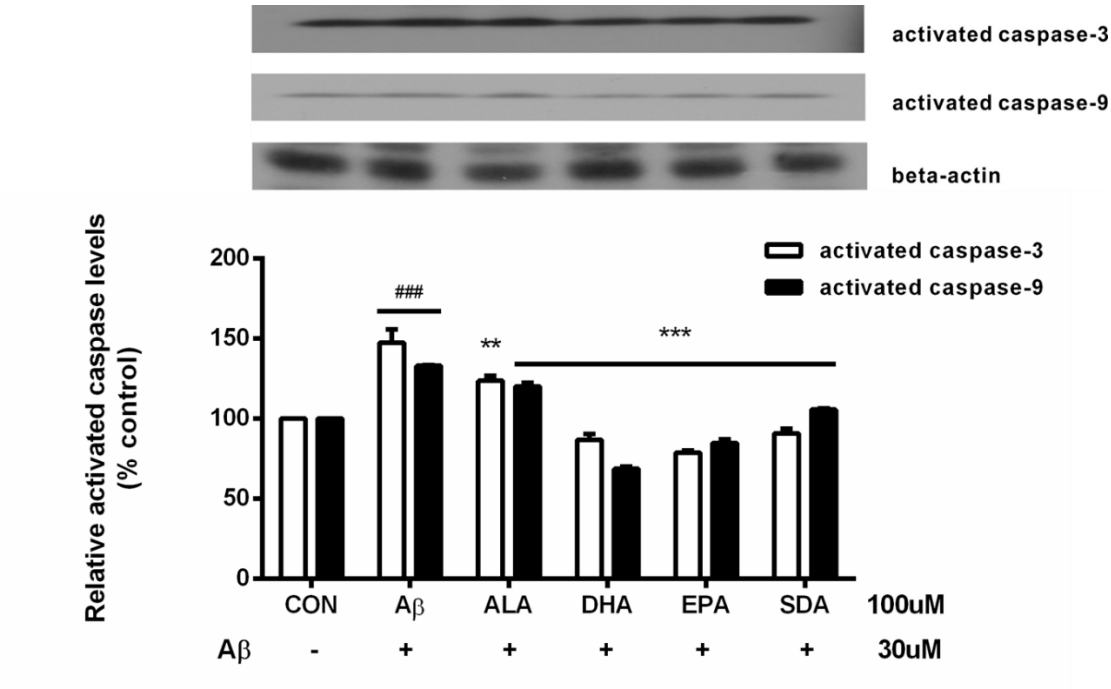

Figure 9 Effect of different ω-3 fatty acids on the protein expression of the activated caspase-3 and activated caspase-9 in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. At the end of the treatment, cells were harvested and activated caspase-3 and activated caspase-9 protein levels were measured by Western blot analysis. The activated caspase-3 and activated caspase-9 determined by Image Studio Lite software were then calculated and graphed. Shown are representative blot and the relative expression of activated caspase-3 and activated caspase-9 were quantified according to the level of β-actin. Values were obtained from three independent experiments and were expressed as the means ± SEM; Data were normalized to the effect of control designated as 100%; $^{###}P < 0.001$, different from BSA-ethanol vehicle treated control cells; $^{**}P < 0.01$, $^{***}P < 0.001$, different from Aβ1-40-treated cells.

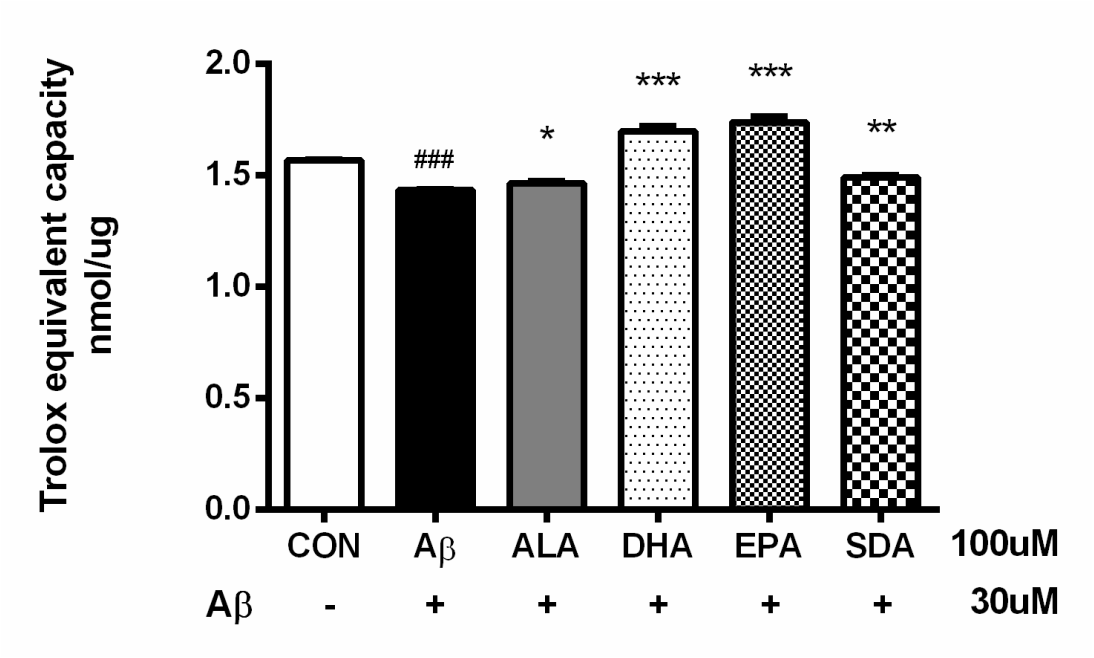

Figure 10 Effect of different ω-3 fatty acids on Total Anti-Oxidant Capacity (T-AOC) in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. The total antioxidant potential of samples was determined spectrophotometrically at 570 nm using a T-AOC assay kit. Values were normalized to the protein content. Data were shown in the unit of nanomoles per microliter Trolox equivalents and expressed as mean ± SEM from three independent experiments. $^{###}P < 0.001$, different from BSA-ethanol vehicle treated control cells; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from Aβ1-40-treated cells.

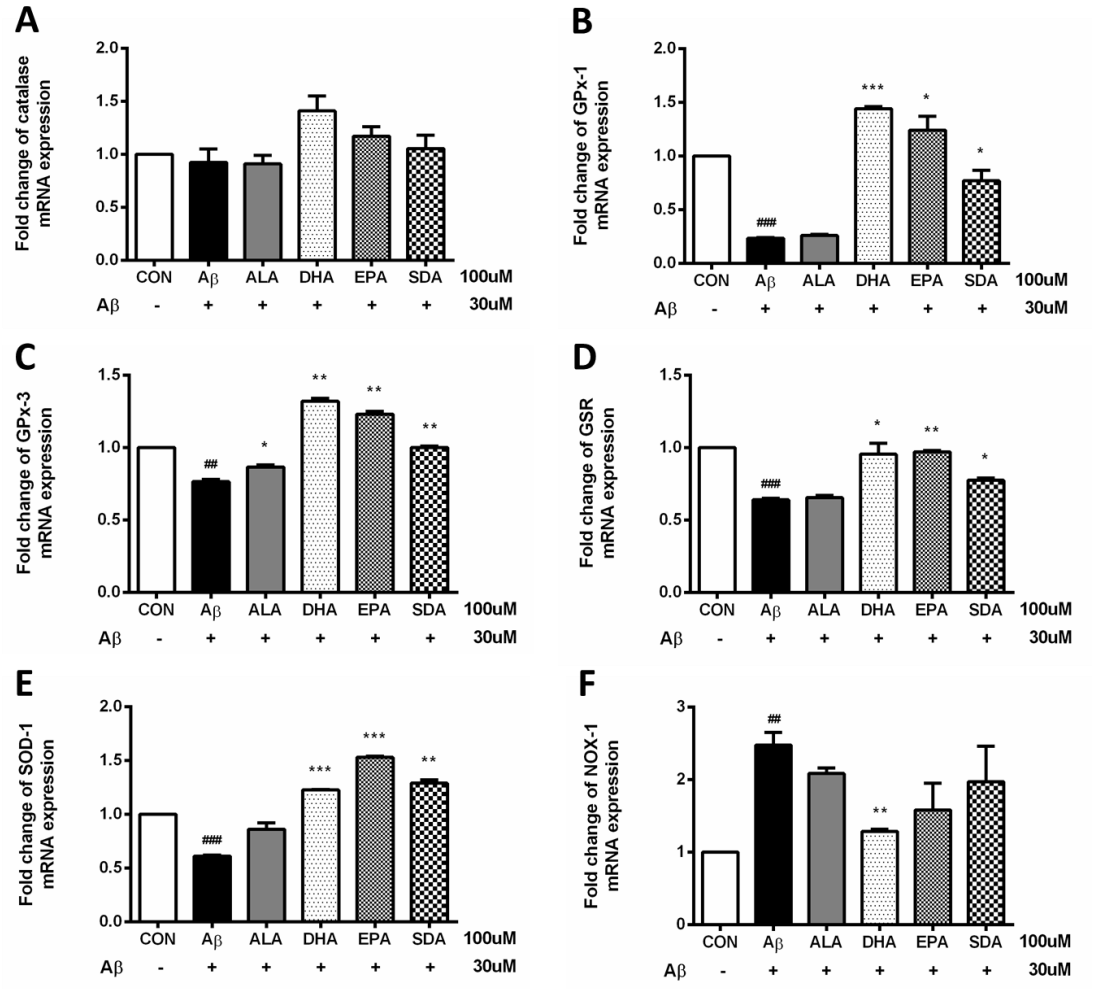

Figure 11 Effects of different ω-3 fatty acids on the expression of anti-oxidant and pro-oxidant genes in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. The mRNA expression of anti-oxidant gene catalase (A), GPx-1 (B), GPx-3 (C), GSR (D), SOD-1 (E) and pro-oxidant gene NOX-1 (F) in H19-7 cells were analyzed by quantitative real-time PCR. Values were obtained from three independent experiments and were expressed as the means ± SEM; Data were normalized to the level of β-actin mRNA. $^{\#\#}P<0.01$, $^{\#\#\#}P < 0.001$, different from BSA-ethanol vehicle treated control cells; *P<0.05, **P < 0.01, ***P < 0.001, different from Aβ1-40-treated cells.

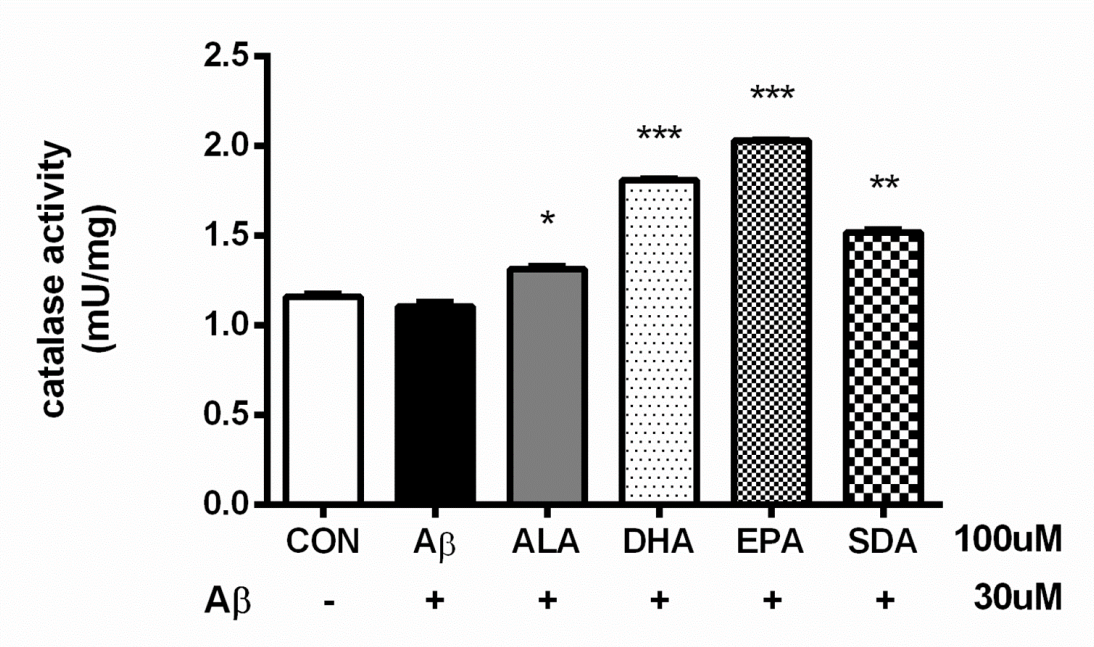

Figure 12 Effect of different ω-3 fatty acids on catalase activity in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. Catalase activity of the samples was determined spectrophotometrically at 570 nm using a catalase activity assay kit. Values were shown as microunit per microgram protein and expressed as mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

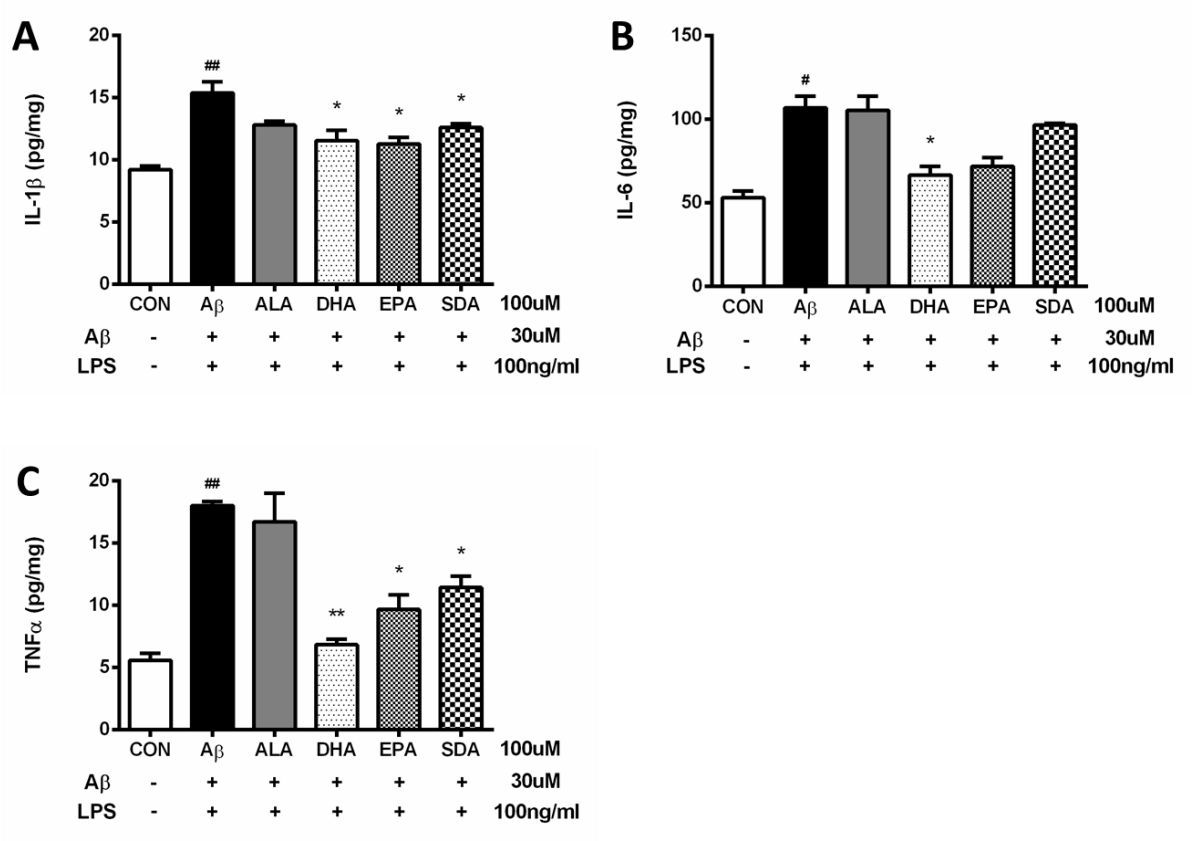

Figure 13 Effect of different ω-3 fatty acids on the release of pro-inflammatory cytokines, IL-1β (A), IL-6 (B), and TNFα (C) in H19-7 cells induced by Aβ1-40 and LPS.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 and 100 ng/ml LPS were added into the medium for another 24 h incubation. The levels of pro-inflammatory cytokines in cell culture supernatant were determined using ELISA kits at 450nm. Values were normalized to the protein content and expressed as mean ± SEM from three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, different from BSA-ethanol vehicle treated control cells; $^{*}P < 0.05$, $^{**}P < 0.01$, different from Aβ1-40-treated cells.

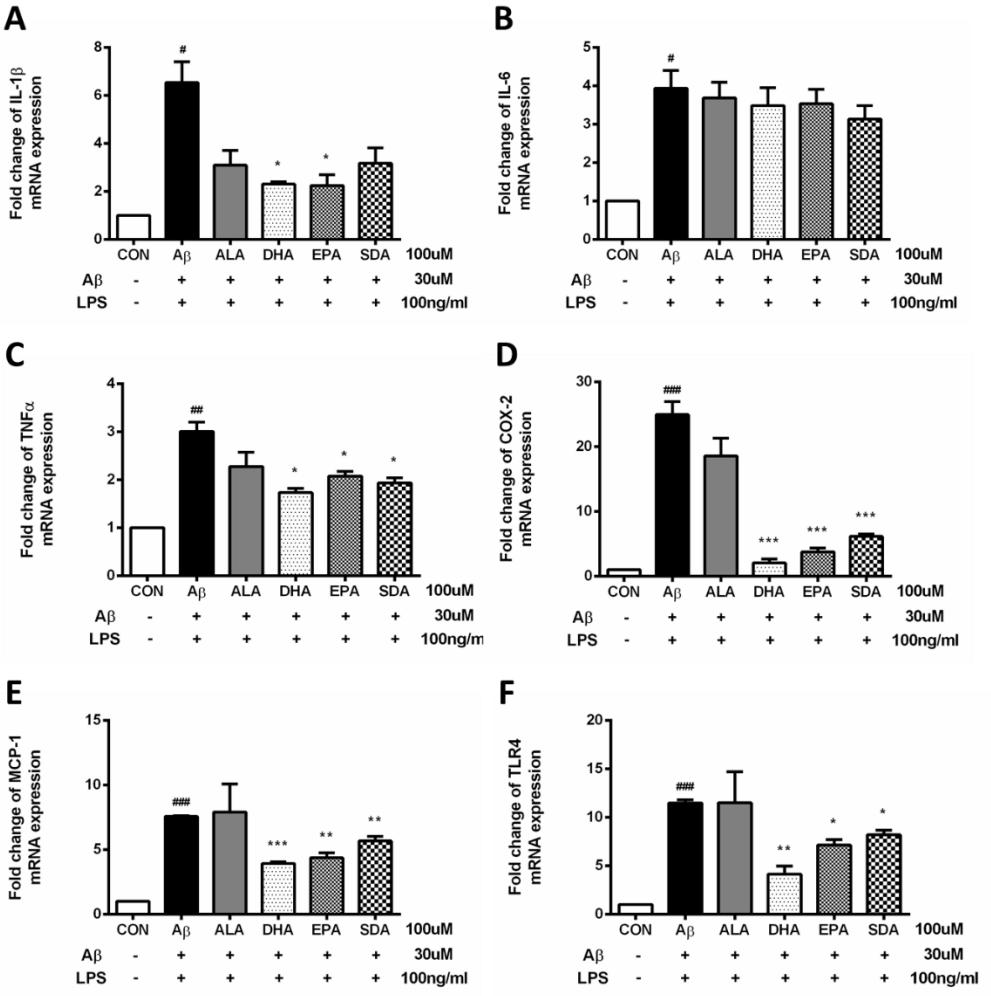

Figure 14 Effects of different ω-3 fatty acids on the gene expression of proinflammatory mediators in H19-7 cells induced by Aβ1-40 and LPS.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 and 100 ng/ml LPS were added into the medium for another 24 h incubation. The mRNA expression of proinflammatory mediators IL-1β (A), IL-6 (B), TNFα (C), COX-2 (D), MCP-1 (E), and TLR4 (F) in H19-7 cells were analyzed by quantitative real-time PCR. Values were obtained from three independent experiments and were expressed as the means ± SEM; Data were normalized to the level of β-actin mRNA. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P < 0.001$, different from BSA-ethanol vehicle treated control cells; *P<0.05, **P < 0.01, ***P < 0.001, different from Aβ+LPS-treated cells.

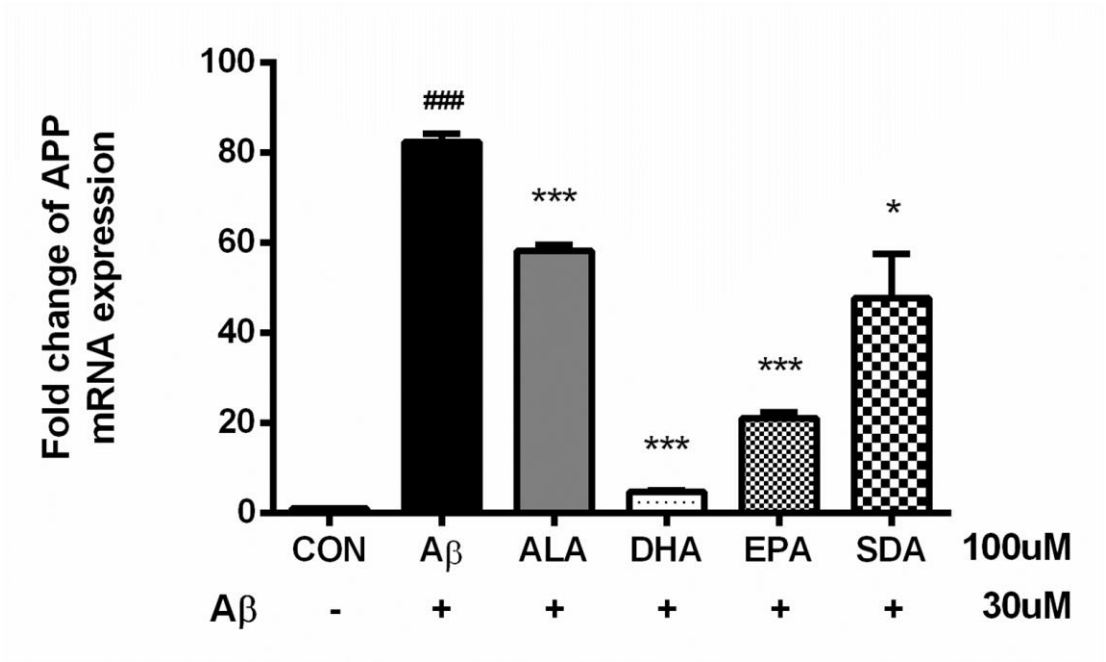

Figure 15 Effects of different ω-3 fatty acids on the gene expression of APP in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. The mRNA expression of APP in H19-7 cells were analyzed by quantitative real-time PCR. Values were obtained from three independent experiments and were expressed as the means ± SEM; Data were normalized to the level of β-actin mRNA. $^{###}P < 0.001$, different from BSA-ethanol vehicle treated control cells; $*P<0.05$, $***P < 0.001$, different from Aβ-treated cells.

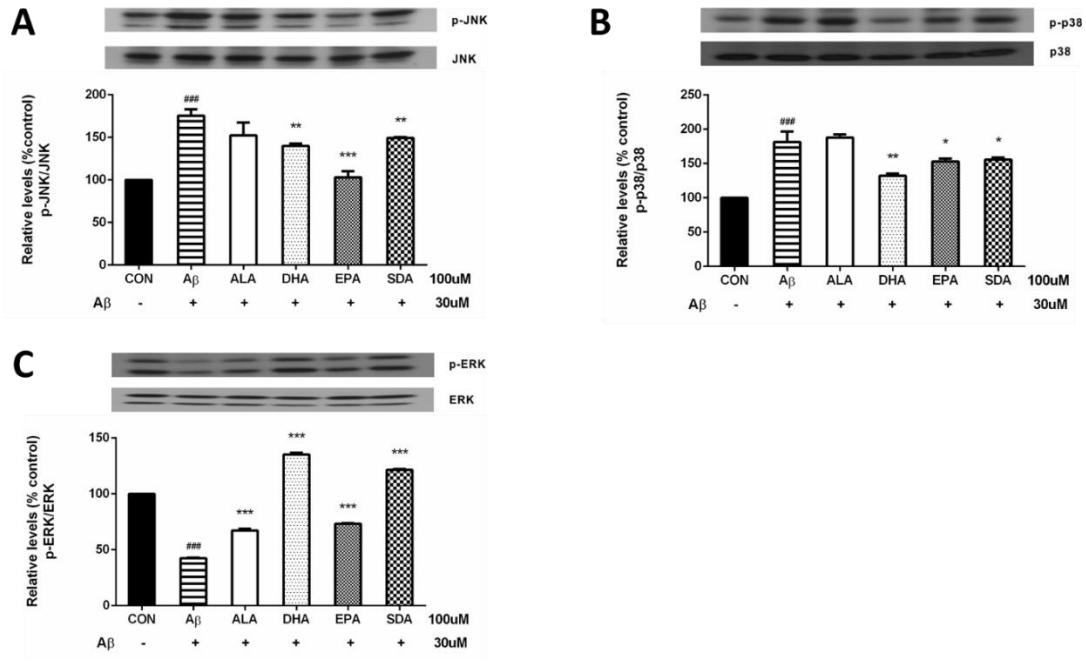

Figure 16 Effect of different ω-3 fatty acids on MAPKs activation in H19-7 cells induced by Aβ1-40.

H19-7 cells were pretreated with ALA, DHA, EPA, SDA, or BSA-ethanol vehicle control in the concentration of 100 μM for 48 h, then 30 μM Aβ1-40 was added into the medium for another 24 h incubation. At the end of the treatment, cells were harvested and MARKs protein levels were measured by Western blot analysis using phospho-specific antibodies for each MAP kinase. The protein levels of MARKs determined by Image Studio Lite software were then calculated and graphed. Shown are representative blot and the relative expression of phospho-JNK (A), phospho-p38 (B), and phospho-ERK (C) were quantified according to the level of each un-phosphorylated forms. Values were obtained from three independent experiments and were expressed as the means ± SEM; Data were normalized to the effect of control designated as 100%; $^{###}P < 0.001$, different from BSA-ethanol vehicle treated control cells; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, different from Aβ1-40-treated cells.

Chapter 5 Omega-3 fatty acids altered the eicosanoid profile in cultured rat hippocampal cells revealed by lipidomics.

5.1 Abstract

Supplementation of long-chain ω-3 PUFA into the human diet contributes to anti-inflammatory properties and benefits for certain chronic inflammatory conditions. It is known that ω-3 PUFAs exert their biologic activities mainly via formation of eicosanoids by competing with ω-6 PUFAs. Generally, ω-3 derived eicosanoids are less potent than analogous ω-6 derived eicosanoids. However, to date, only a few studies have investigated the overall effects on eicosanoid production by ω-3 fatty acids, mainly DHA and EPA; no study has been conducted on SDA treatment. Therefore, in order to better understand how ω-3 fatty acids work after incorporation into the cell membrane, the present study employed lipidomics, a large-scale study of pathways and networks of cellular lipids in biological systems, to characterize the changes in eicosanoid profile in H19-7 rat hippocampal cells upon treatment of different ω-3 fatty acids. Our results showed that ω-3 fatty acids affect the production of eicosanoids by LOX, COX, CYP, and non-enzymatic autoxidation pathways. In addition, we confirmed that ω-3 fatty acids are natural anti-inflammatory compounds that can convert to many anti-inflammatory eicosanoids under normal conditions. While, we also observed increased production of many pro-inflammatory metabolites by ω-3 fatty acids, especially by EPA. Further, treatment of DHA can increase the ROS production. In summary, the effects on eicosanoid production are consistent among all four ω-3 fatty acids and the efficacy of anti-inflammatory potent is DHA&EPA > SDA >> ALA. Particularly, the changing pattern of eicosanoids affected by SDA treatment is most close to the

pattern by EPA, which confirmed the proposal that SDA is the surrogate for EPA. Importantly, compared to EPA, SDA is much less potent in increasing the production of anti-inflammatory metabolites, but also much less potent in increasing the production of pro-inflammatory metabolites, indicating SDA may have advantages over EPA. Moreover, except being a surrogate for EPA, it is indicated that SDA has its unique roles in biosynthesis of eicosanoids, such as decreased PGD1 level, suggesting that SDA may have its own specific biological functions different from DHA and EPA. Finally, this lipidomics study of ω-3 fatty acids is valuable and provides inspiration for future studies, especially in the area of chronic inflammatory diseases, the area of cardiovascular system where ω-3 fatty acids act as vasodilator, and the area of host immune defense.

5.2 Background

Supplementation of long-chain ω-3 PUFA into the human diet contributes to anti-inflammatory properties and benefits for certain chronic inflammatory conditions, such as obesity, CVD, diabetes, rheumatoid arthritis, AD, and cancer (4, 226, 294, 410, 411). However, the molecular mechanisms responsible for these benefits are unclear. The ω-3 PUFAs exert their biologic activities mainly via formation of bioactive lipid mediators, known as eicosanoids (412). On the other hand, ω-3 PUFAs compete with ω-6 PUFAs for incorporation into membrane phospholipids and for use as substrates for the same enzymes, thereby affect ω-6 derived eicosanoids (10). Generally, ω-3 derived eicosanoids are less potent than analogous ω-6 derived eicosanoids (413), therefore, the majority of studies nowadays have focused on the downstream eicosanoid biosynthesis.

Eicosanoids constitute a diverse class of bioactive signaling molecules and are part of the innate and adaptive immune systems, in which eicosanoids mediate inflammation, pain, fever, vasodilation, and chemotaxis (414). Eicosanoids are formed via the same three enzymatic pathways: LOX, COX, and cytochrome P450 (CYP). As substrates, ω-3 and ω-6 PUFAs compete for these enzymes and their downstream metabolic products are connected to disease etiology. Eicosanoids derived from ω-6 AA, such as prostaglandin (PG) 2 series, thromboxane (TB) 2 series, and leukotriene (LT) 4 series are found to be proinflammatory and linked to the incidence of some chronic inflammatory disease (415, 416). Comparatively, data suggest that ω -3 derived eicosanoids, such as PG 3 series, TB 3 series, LT 5 series, resolvins, protectins, and maresins possess anti-inflammatory and proresolving bioactivity, which allowing them to exert beneficial health effects in many chronic inflammatory diseases (417, 418). However, to date, only a few studies have investigated how ω-3 fatty acids affects the overall profile of downstream eicosanoid biosynthesis, and most of the research performed has focused on DHA and EPA (419, 420). No study has been conducted by SDA treatment.

Lipidomics is the large-scale study of pathways and networks of cellular lipids in biological systems (421). Lipidomics is a relatively recent research field that has been driven by rapid advances in technology. Basically, lipidomics is composed of two distinct approaches, electrospray ionization coupled with single stage or tandem mass spectrometry approach and a liquid chromatography-mass spectrometry (LC/MS) (422). Lipidomics is now considered as the best method for analysis of lipid extracts since it allows infusion of lipid mixtures into the mass spectrometer and analysis of all the molecules at one time (423). By lipidomics, all the lipids, even tiny amount (pmol/ml) in complex mixtures are identified and quantified simultaneously and with high accuracy, which provides global information about cellular lipid profile. Although the sample

handling and enormous data analysis limit the application of lipidomics, it is still rapidly developed and has been widely used in measuring fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, and prenols (422).

Lipid profiling is most used to provide useful information on the basal lipidome status of healthy volunteers or patients with diabetes, CVD, metabolic disorders, and obesity (422). In addition, lipidomics is also employed in pharmaceutical research, such as to determine response to drug therapy (423). In ω-3 intervention trials, dietary supplementation of DPA and EPA has been found to increase the levels of resolvin, maresin, and some other lipid mediators in plasma (424). In animal studies, people have determined the effects of ω-3 supplementation by lipidomics on hepatic lipogenesis (425) and brain composition (426). It has been showed that feeding of fish oil to rats results in their incorporation into brain phospholipids, the extent of which is lower in the striatum as compared with cortex and hippocampus (426). We have demonstrated a protective effect by ω-3 fatty acids on Aβ-induced neurotoxicity in H19-7 hippocampal cells (Chapter 4). We demonstrated the anti-oxidative and anti-inflammatory properties of ω-3 fatty acids (Chapter 4). In order to better understand how ω-3 fatty acids work and what happened after incorporation into the cell membrane, the present study employed lipidomics and aimed to characterize the changes in eicosanoid profile in response to 48-hour treatments of different ω-3 fatty acids in H19-7 rat hippocampal cells.

5.3 Materials and methods

5.3.1 Cell culture

The H19-7 cell line was derived from hippocampi dissected from embryonic day 17 (E17) Holtzman rat embryos and immortalized by retroviral transduction of temperature sensitive tsA58

SV40 large T antigen. The cells were generously provide by Dr. Ramesh Jeganathan. All cells were cultured in poly-D-lysine-coated culture dishes and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin-streptomycin (Sigma), 0.001mg/ml puromycin (Sigma), and 0.2 mg/ml G418 (Sigma) in a humidified incubator at 34°C with 5% CO2.

5.3.2 Fatty acid treatment

Fatty acids (ALA, SDA, EPA, and DHA) were purchased from Matreya LLC, (Stage College, PA). Stock solutions of fatty acids were in ethanol and further diluted in DMEM containing 1.5% of fatty acid-free bovine serum albumin (BSA). Fatty acids were delivered to the cells as fatty acid/BSA complexes. BSA-ethanol vehicle was used a control. After incubation at 37ºC for 1h, fatty acid-supplemented medium (50 or 200 μM) or BSA-ethanol vehicle control was applied to H19-7 hippocampal cells for two days. Fatty acids were delivered to the cells as fatty acid/BSA complexes. BSA-ethanol vehicle was used a control. At the end of the treatment, cell culture supernatant was collected and send to the LIPID MAPS Lipidomics Core at the University of California, San Diego for a comprehensive eicosanoid panel.

5.3.3 Comprehensive Eicosanoid penal

Eicosanoids are analyzed by UPLC/MS/MS and are fully quantitated by comparison with authentic standards. The comprehensive eicosanoid panel is a global eicosanoid profile consisting of 157 eicosanoids provided by Lipidomics and Metabolomics Analytical Services at the University of California, San Diego. Briefly, 100 μL internal standard mix was added to each 200 μL cell supernatant sample. Eicosanoids were extracted using solid-phase extraction with strata-x polymeric reverse phase columns (8B-S100-UBJ Phenomenex). The following was added to each

column by order: 100% MeOH, 100% H_2O, sample, 10% MeOH, and 100% MeOH for elution. The elution was then dried down using speed-vac. After drying, the samples were taken up in 100 µL Buffer A (63% H_2O, 37% ACN, and 0.02% acetic acid). 10 µL of the sample was injected into UPLC column (Waters ethylene bridged hybrid, 130 Å, 1.7 µM, 2.1x100mm). Eicosanoids were subsequently detected using a mass spectrometer (Sciex 6500 Qtrap).

5.3.7 Statistical analysis

All data are presented as mean ± SEM. The statistical significance of differences between groups was determined by one-way analysis of variance (One-way ANOVA) and Student's t-test (two-tailed). The results were considered to be significant when the value of P was < 0.05. Figures were produced by GraphPad PrismTM version 6.01 (GraphPad software, San Diego, CA).

5.4 Results and Discussion

5.4.1 ALA-derived eicosanoid production in response to ω-3 fatty acids

HOTrEs including 9-HOTrE and 13-HOTrE are monohydroxy PUFAs derived from ALA by the action of 5-LOX and 15-LOX, respectively. The biological role of 9-HOTrE is still unknown but it may be involved in natural senescence (427). 13-HOTrE has been reported to have anti-inflammatory properties as evidenced by reduction of IL-1β-induced MMP expression in human chondrocytes and may play a role in inflammatory joint diseases (428). In the present study, as shown in Figure 17A, supernatant concentration of 9(S)-HOTrE was significantly increased in H19-7 cells, not only by treatment with ALA (462-fold), but also by treatment with DHA (3.0-fold), EPA (2.4-fold), and SDA (3.2-fold) compared to control. While, as shown in Figure 17B, supernatant concentration of 13(S)-HOTrE was only dramatically increased by ALA; 13(S)-HOTrE was not even detected by control, EPA, and SDA treatment. The different regulation

between the two HOTrEs indicates that the effects of ω-3 fatty acids on eicosanoid biosynthesis may rely on the different LOX pathways. Previous study has demonstrated a positive correlation between ALA levels in erythrocyte membranes and the plasma levels of 9-HOTrE and 13-HOTrE in the hyperlipidemic men (429). Renal levels of HOTrEs were also shown to increase in rats when dietary ALA was provided (430). These results suggest that ω-3 fatty acids has anti-inflammatory effects in neurons and may be beneficial in cell survival, inflammatory joint diseases, and dyslipidemia.

5.4.2 EPA-derived eicosanoid production in response to ω-3 fatty acids

HEPEs are produced by LOX pathway of EPA. In general the physiological effects of HEPEs are unclear; however, it has been suggested that HEPEs compete with AA as a substrate for 5-LOX in the formation of leukotrienes and may have anti-inflammatory effects in the resolution phase of inflammation (431). The level of 5-HEPE, whose biological effects were lower compared to AA-derived metabolites (432) has been found to increase 24-hour after cardiac surgery in a lipidomics study (433). Dietary supplementation with ω-3 fatty acids have been demonstrated to reduce the pro-inflammatory mediators (HETEs, bicycle-PGF2, and 5-iPF2α-IV), while increasing the levels of anti-inflammatory mediators (5-HEPE, 15-HETrE, and 17-HDoHE) in rat plasma (434). Our results showed that the supernatant concentration of 5(S)-HEPE was significantly increased in H19-7 cells by treatment with DHA (10-fold), EPA (45-fold), and SDA (7.2-fold) compared to control (Figure 18A). HEPEs, including (5-, 8-, 9-, 12-, 18-) has been proven to act as ligands for PPARs, which influence fatty acid oxidation, TG levels, glucose uptake and insulin sensitivity (435, 436). Among these HEPEs, 8-HEPE has been found most effective and enhance adipogenesis and glucose uptake (436). Our results showed that the concentration of 8(S)-HEPE was significantly increased by treatment with DHA (3.8-fold), EPA (48-fold), and

SDA (1.9-fold) compared to control (Figure 18B). As shown in Figure 18C, the concentration of 9(S)-HEPE was significantly increased by treatment with DHA (9.0-fold), EPA (45-fold), and SDA (3.9-fold) compared to control. 12-HEPE has been found to elicit an inhibitory effect on platelet aggregation (437) and U46619-induced rat aorta contraction (438) with the same potency as 12-HETE (439). In addition, 12-HEPE has been shown to attenuate serotonin (5-HT) release mediated by AA and collagen in a dose-dependent manner (440). 12-HEPE was also demonstrated to be associated with an anti-inflammatory profile in a study which assess the impact of EPA on rabbit alveolar macrophages (441). Dietary EPA has been found to increase skin 12-HEPE in humans (442). Incorporation of EPA in to glycerophospholipids has been found to increase 12-HEPE in isolated brain cells from rainbow trout (443). Our results showed that the concentration of 12(S)-HEPE was significantly increased by treatment with DHA (1.7-fold), EPA (20-fold), and SDA (1.2-fold) compared to control (Figure 18D). Conversely, ALA treatment significantly decreased 12(S)-HEPE level by 27% (Figure 18D). Moreover, 12-HEPE and 15-HEPE have been found to suppress the development of endometriotic lesions (444). Animal study has demonstrated the potential anti-inflammatory property of 15-HEPE in skin disorders (431). Our results showed that the concentration of 15(S)-HEPE was significantly increased by treatment with DHA (2.6-fold) and EPA (11-fold) compared to control (Figure 18E). The different regulation by each ω-3 fatty acid indicates that the metabolism and effects of DHA, EPA, and SDA tend to be consistent, while the ALA may sometimes has opposite effects on eicosanoid biosynthesis. These results suggest that the anti-inflammatory effects of DHA, EPA, and SDA may be stronger than ALA and by these anti-inflammatory effects, ω-3 fatty acids may be beneficial in energy metabolism, insulin sensitivity, platelet aggregation, CVD, neuron function, and inflammatory skin disorders.

PGD3 and PGE3 are produced by the metabolism of EPA via the COX pathway. PGD3 has been found to modulate sympathetic nerve transmission by reducing norepinephrine release (445), whose concentration was found decreased in various regions of the Parkinson's disease brain (446). PGD3 has also been found to decrease systemic blood pressure in rats and inhibit ADP-induced platelet aggregation in human (447). When applied to the eyes of rabbits, PGD3 and PGE3 lowered intraocular pressure without causing ocular inflammation, which often seen in PGD2 (448, 449). In IgA nephropathy patients, fish oil supplementation was shown to increase the serum concentration of PGD3 (450). Treatment of ω-3 fatty acids were found to increase PGE3 level and inhibit COX-2-mediated tumor invasion in brain-melanoma cells (451). In the present study, as shown in Figure 19A&B, PGD3 and PGE3 were not detectable in the supernatant of cells without treatment; however, treatment of EPA dramatically increased both PGD3 and PGE3 level; treatment of SDA also has similar, but less potent effects on the levels of PGD3 and PGE3; and ALA increased PGD3 level, while DHA increased PGE3 level. These results suggest that the anti-inflammatory effects of ω-3 fatty acids, especially EPA and SDA may rely on their conversion to PGD3 and PGE3, therefore ω-3 fatty acids may be beneficial in neurodegenerative disease, blood pressure, intraocular pressure, platelet aggregation and cancer. 11(S)-HEPE is a monohydroxy fatty acid derived from EPA, theoretically by the action of COX. As shown in Figure 19C, the concentration of 11(S)-HEPE was significantly increased by treatment with ALA (1.4-fold), DHA (4.3-fold), EPA (58-fold), and SDA (3.3-fold) compared to control.

5.4.3 DPA-derived eicosanoid production in response to ω-3 fatty acids

17k DPA is a metabolite of LOX-mediated oxidation of DPA. 17k DPA has been shown to activate Nrf2-dependent antioxidant gene expression, to act as a PPARγ agonist, and to inhibit pro-inflammatory cytokine and NO production (452). In the present study, as shown in Figure 20,

the level of 17k DPA was not detectable in the supernatant of cells without treatment; however, treatment of DHA dramatically increased 17k DPA level; treatment of ALA, EPA, and SDA also has similar but much less potent effects on the levels of 17k DPA. These results suggest that ω-3 fatty acids, especially DHA have both anti-oxidative and anti-inflammatory effects.

5.4.4 DHA-derived eicosanoid production in response to ω-3 fatty acids

HDoHEs are autoxidation product of DHA. The function of HDoHEs is not clear; however, 14-HDoHE is precursor of maresin1 (453) and 17-HDoHE is precursor of resolvin D and protectin D (454). Maresin1 possess potent anti-inflammation and is involved in allergic reactions, would healing, and abating neuropathic pain (455). Protectin D, also known as neuroprotection D, whose level was reduced in AD, was shown to have neuroprotective activity by reducing inflammation and apoptosis (456). In addition, HDoHEs can further convert to oxoDHA, which often induce anti-inflammatory and anti-proliferative responses (457). 14-HDoHE was determined to directly blunt the IL-6 mRNA expression (457). 17-HDoHE and neuroprotection D1, as well as AA-derived 12-HETE and 15-HETE have been shown to improve severe influenza (458). 11- and 17-HDoHEs were shown to inhibit U-46619-induced rabbit and rat aortic smooth muscle contraction (438). Treatment of DHA or ω-3 fatty acids was found to increase 7-HDoHE level in the membrane of RAW264.7 macrophages (459), 4-HDoHE in plasma and aorta samples of mice (460), and 7-, 10-, 17-HDoHEs in brains of aged rats (461). On the other hand, HDoHEs are potential markers of oxidative stress in brain and retina where DHA is an abundant PUFA. The action of 19,20-DiHDPA is still poorly understood.

Our results showed that treatment of DHA significantly increased 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, 20-HDoHEs and 19,20-DiHDPA by 11~41 folds when compared with control (Figure 21A-K). Treatment of ALA, EPA, and SDA also significantly increased 4- and 7-HDoHEs

by 2.6-4.6 folds compared to control group (Figure 21A&B). Moreover, EPA treatment increased 8-HDoHE by 2.1-fold (Figure 21C), while SDA treatment increased 10-HDoHE by 1.5 fold (Figure 21D). These results indicate that DHA-derived HDoHEs are poorly affected by other ω-3 fatty acids. Further, it suggests that ω-3 fatty acids, especially DHA have anti-inflammatory effects and may be beneficial in influenza, but also can induce oxidative stress in neurons.

5.4.5 LA-derived eicosanoid production in response to ω-3 fatty acids

The most prevalent LA-derived oxylipins are the 9- and 13-HODEs. Oxidized LDL contains significant amount of esterified 9- and 13-HODEs (462, 463). Generally HODEs have been shown to have anti-inflammatory and anti-proliferative effects (464-466) and play a role in platelet activity (467, 468). However, it becomes controversial for different HODE members. 9-HODE has been ascribed a proinflammatory effect by activating G2A, which mediates intracellular calcium mobilization and JNK activation in human skin under oxidative conditions (469). In a rat wound-healing model, 9-HODE has showed a robust proinflammatory effect (470). While 13-HODE is an agonist for PPARγ and plays an anti-inflammatory role (471). 13-HODE is the principle hydroxylated fatty acid in human psoriatic skin scales (472). HODEs are also involved in macrophage apoptosis, a key process in atherogenesis. The number of cells was reduced within 24 h following treatment with 9-HODE or 13-HODE with an up-regulation of caspase activity, suggesting a pro-apoptotic role of HODEs (473). Anyway, in this same study, treatment of LA or ALA didn't affect macrophage apoptosis. Renal levels of the HODEs in rat were higher when more dietary LA was provided (430). Under oxidative stress, HODEs appear to convert to oxoODEs (474, 475). Unlike 9-HODE, 9-oxoODE has anti-inflammatory property. Treatment with 9-oxoODE was shown to diminish oxidant-induced macrophage inflammation as indicated by decreased production of TNFα (476). In the present study, as shown in Figure 22A,

supernatant concentration of 9-HODE was significantly increased in H19-7 cells by treatment with ALA (1.8-fold), DHA (2.2-fold), EPA (1.5-fold), and SDA (2.0-fold) compared to the control group. As shown in Figure 22B, concentration of 9-oxoODE was only significantly increased by EPA (1.3-fold). These results indicate that EPA may be more anti-inflammatory then other ω-3 fatty acids by promoting the conversion of pro-inflammatory 9-HODE to anti-inflammatory 9-oxoODE. As shown in Figure 22C, concentration of 13-HODE was only significantly increased by ALA (1.9-fold). ALA is supposed to have more regulation on LA-derived oxylipins since it directly compete enzymes with LA. These results suggest that ω-3 fatty acids have not only anti-inflammatory effects but also pro-inflammatory and pro-apoptotic effects by converting to regulating LA-derived oxylipins.

Through the enzymatic activities of CYP450, leukotoxin are generated from LA. This unstable compound is then rapidly degraded to epoxides, mainly 9,10-diHOME and 12,13-diHOME. Leukotoxin promotes mitochondrial dysfunction, vasodilation, and apoptosis. In renal proximal tubular cells, 9,10-diHOME has been directly implicated as the cytotoxic agent responsible for cell death (477). By disrupting mitochondrial function, increased levels of diHOMEs were found to be associated with acute respiratory distress syndrome, pulmonary edema, vasodilation, and cardiac failure in animal models (478-480). However, in renal cortical mitochondria, the conversion of leukotoxin to 12,13-DiHOME appears to be part of the detoxification pathway that prevents mitochondrial dysfunction (481). In the present study, treatment of ω-3 fatty acids showed no effect on the level of diHOMEs, except DHA slightly increased the concentration of 9,10-diHOME (Figure 23). These results suggest that ω-3 fatty acids has little or no effect on the production of the cytotoxic diHOMEs, indirectly exert their protective effects against leukotoxin toxicity and cell death.

5.4.6 GLA-derived eicosanoid production in response to ω-3 fatty acids

13-HOTrE(γ) is the 15-LO product of GLA. The biological function of 13-HOTrE(γ) is not known. In the present study, treatment of ω-3 fatty acids showed no effect on the level of 13(S)-HOTrE(γ) (Figure 24).

5.4.7 DGLA-derived eicosanoid production in response to ω-3 fatty acids

8-HETrE and 15-HETrE are produced by LOX from DGLA. The biological activity of 8-HETrE has not been well characterized. 15-HETrE is believed to be anti-inflammatory and have anti-proliferative effects. 15-HETrE is a potent inhibitor of 5-LOX which catalyze the conversion of AA to LTB4 and EPA to LTB5 (482). Oral DHA and EPA supplementation was shown to lower plasma 15-HETrE level in healthy young adults (483). A study has shown that the application of DHA to pig skin induced hyperproliferation as enhanced expression of anti-apoptotic Bcl-2 but reduced expression of apoptotic caspase-3; however, application of 15-HETrE, on the other hand, reversed DHA-induced epidermal hyperproliferation via the modulation of apoptosis (484). In the present study, as shown in Figure 25A, supernatant concentration of 8(S)-HETrE was significantly increased in H19-7 cells by treatment with DHA (2.9-fold), EPA (2.3-fold), and SDA (2.7-fold) compared to the control. As shown in Figure 25B, treatment of ω-3 fatty acids showed no effect on the level of 15(S)-HETrE.

PGD1 is the D-series metabolite of DGLA. PGD1 is an inhibitor of ADP-induced platelet aggregation in humans about 10% as potent as PGD2 (447), suggesting an anti-inflammatory property as 1-series (485). PGD1 has been found to prevent the development of atopic dermatitis in mice (486). In the present study, as shown in Figure 26, the concentration of PGD1 was significantly decreased by treatment with SDA while other ω-3 fatty acids showed no effects. This

result indicates that SDA may be somehow "bad" by reducing the production of anti-inflammatory PGD1, and also suggest that except acting as a surrogate for EPA, SDA may has its own unique role and mechanism in biological activities.

5.4.8 AA-derived eicosanoid production in response to ω-3 fatty acids

AA is the major precursor of eicosanoids. By 5-LOX, 12-LOX, and 15-LOX, AA can convert to 5-HpETE, 12-HpETE, and 15-HpETE, respectively. HpETEs are unstable and will rapidly convert to different stable compounds. 5-HpETE converts to LT series 4 and 5-HETE. In the present study, our results showed that the concentration of LTB4 was only detectable and relatively high in EPA-treated cell (Figure 27A). This result is intriguing since in a previous study, EPA from diet acted as a competitive inhibitor of AA conversion to LTB4 (487). As a pro-inflammatory cytokine, LTB4 promotes a number of leukocyte functions including aggregation, stimulation of ion fluxes, enhancement of lysosomal enzyme release, improvement of superoxide anion and ROS production, increase of oxidative stress, chemotaxis, and chemokinesis (488). 5-HETE family play an important role in host immune defense but also implicated in aberrant pro-inflammatory disease, such as arthritis and allergy. 5-HETE was also shown a potent survival factor for human prostate cancer cells (489). Fish oil supplementation of the human diet has been shown to result in decreased production of 5-HETE by inflammatory cells (490, 491). However, our study shows an opposite results. As shown in Figure 27B, supernatant concentration of 5-HETE was significantly increased in H19-7 cells by treatment with ALA (1.9-fold), DHA (5.4-fold), EPA (5.6-fold), and SDA (5.0-fold) compared to the control cells. This result indicates that ω-3 fatty acids promote the production of AA-derived proinflammatory 5-HETE in neurons, although they are anti-inflammatory on the whole. 5-HETE is further metabolized to 5,15-diHETE. 5,15-diHETE is proinflammatory and potentiates the degranulation of human polymorphnuclear

leukocytes (492, 493). Interestingly, our results showed that supernatant concentration of 5,15-diHETE was significantly increased in H19-7 cells by DHA (2.0-fold) and EPA (45-fold) compared to the control cells (Figure 27C). This result indicates that EPA promotes the production of proinflammatory 5,15-diHETE in neurons dramatically, as much as 45 folds. However, SDA showed no effect on the 5, 15-diHETE production, indicating that SDA may not only be a substitute for EPA, but may be more anti-inflammatory than EPA in some way.

12-HpETE is the precursor of 12-HETE and hepoxilin 3-series. Our results showed that treatment of ω-3 fatty acids showed no effect on the level of 12-HETE (Figure 27D). The properties of 12-HETE is controversial. 12-HETE was shown to exert chemoattractant actions and pro-inflammatory effects in various cell types and skin disease (482, 494). On the other hand, 12-HETE was also shown to block the inflammatory response in arthritis by reduced IL-6 production in TNFα-stimulated macrophages (495). Tetranor 12-HETE is the β-oxidation product resulting from peroxisomal metabolism of 12-HETE in numerous tissues (496). No biological function has yet been determined for tetranor 12-HETE. Some data indicate tetranor 12-HETE may play a role in controlling the inflammatory response in injured corneas (497). As shown in Figure 27E, our results showed that supernatant concentration of tetranor 12-HETE was significantly decreased by ALA, DHA, EPA, and SDA by 17%, 40%, 49%, and 48%, respectively. It might be possible that ω-3 fatty acids compete with 12-HETE as substrate for β-oxidation, therefore inhibit tetranor 12-HETE production. As shown in Figure 27F, the supernatant concentration of HXB3 was significantly increased in H19-7 cells by DHA (1.7-fold) and SDA (1.5-fold) compared to the control cells. This proved again that SDA may have its own effects except being the substitute for EPA. HXB3 is proposed to possess proinflammatory actions in stimulating human neutrophil chemotaxis and increasing the permeability of skin capillaries in inflammatory skin diseases (498).

15-HpETE is the precursor of 15-HETE. Although derived from AA, 15-HETE is generally believed with anti-inflammatory property. 15-HETE was shown to inhibit the formation of the pro-inflammatory 12-HETE in the epidermis (494). In addition, 15-HETE and 12-HETE were found to block the inflammatory response in arthritis by decreased IL-6 production in TNFα-stimulated macrophages (495). On the other hand, 15-HETE appear to act as tumor suppressor. 15-HETE could bind with and activate PPARγ, therefore inhibit the growth of cultured cancer cells (499). Decreased cellular 15-HETE production that occurs in human prostate may be one mechanism by which human cancers avoid apoptosis and thereby progress and spread (500). In the present study, we found that the supernatant concentration of 15-HETE was significantly increased by ALA (1,7-fold), DHA (2.0-fold), EPA (1.9-fold), and SDA (3.3-fold) compared with the control cells (Figure 27G). This may somehow explain our previous finding (Chapter 4) that SDA was most effective fatty acid to improve the cellular viability in Aβ-induced H19-7 cells. These results confirmed that ω-3 fatty acids are involved in chronic inflammatory conditions, such as arthritis and cancer.

In COX pathway, PGG2 is the first intermediate from AA. Under normal condition, PGG2 is quickly metabolized to give PGH2, which serves as the key precursor to the PG 2-series and thromboxanes (501). Although being an AA-derived thromboxane, TXB2 is not pro-inflammatory since it is the inactive form of TXA2, which is the major component of AA-derived proinflammatory thromboxanes. The effect of ω-3 PUFAs on TXB2 production remains controversial. Several studies suggested that marine-derived ω-3 PUFAs resulted in decreased TXB2 in plasma (502-504), while some studies showed increased TXB2 production (505). Our results showed no effect on the TXB2 concentration by treatment with any ω-3 fatty acid.

AA-derived PG 2-series promote swelling, inflammation, clotting and dilation at sites of injury. There are four principal bioactive prostaglandins-PGE2, PGD2, PGI2, and PGF2α, other

PG 2-series are much less potent. In the present study, only EPA treatment significantly increased the concentration of PG 2-series, including PGJ2, dhk PGD2, PGA2, PGB2, and PGF2α (Figure 28B-F); Other ω-3 fatty acids showed no effect on PG 2-series. PGJ2 was shown to inhibit platelet aggregation (447) and have antitumor and anti-viral activities (506). A study provide evidence that dhk PGD2, 11,12-diHETrE, and 20-COOH AA are the leading eicosanoid candidate biomarkers for the noninvasive diagnosis of NASH (507). PGA2 has been shown some antiviral and antitumor activity (508) and acting as a vasodilator with natriuretic properties (509). PGB2 has been found to increase pulmonary blood pressure in animal (510). PGF2α causes contraction of vascular, bronchial, intestinal, and myometrial smooth muscle, and also exhibits potent luteolytic activity (511).

12-HHTrE is an unusual product of the COX pathway and one of the primary arachidonic acid metabolites of the human platelet (512). The biological role of 12-HHTrE is uncertain. Our results showed a significant increase in the level of 12-HHTrE by treatment of EPA (2.0-fold) and SDA (2.0-fold) (Figure 28G).

HETEs and diHETrEs are biologically active eicosanoids that result from the metabolism of AA by CYP pathway. It is now widely recognized that HETEs have important physiological and pathological functions that modulate ion transport, renal and pulmonary functions, vascular tone and reactivity, and inflammatory and growth responses (513). HETEs are strongly involved in the pathogenesis of the CVDs, and inhibiting the formation of mid-chain HETEs has been reported to confer a protection against different cardiac hypertrophy and hypertension models (514). In the present study (Figure 29), we found that ALA treatment has no effect on the production of AA-derived HETEs by CYP pathway. Treatment of DHA, EPA, and SDA all dramatically increased the concentration of 8-, 9-, 17-HETEs. Interestingly, SDA itself

significantly increased 11-HETE concentration and dramatically decreased the concentration of 16-HETE to “not detectable”. These results suggest that SDA may play different roles from DHA and EPA in CVD. 20-COOH-AA is the major metabolite of 20-HETE. It has been demonstrated that 20-COOH-AA is a potent dilator of mouse basilar artery (515). The same research group later demonstrated that 20-COOH-AA is also a dual activator of PPARα and PPARγ (516). Our results showed that only EPA treatment significantly increased the concentration of 20-COOH-AA by 3.1 folds, suggesting a specific role of EPA in blood pressure and cell survival. DiHETEs are found to be anti-inflammatory (517, 518). A mouse study has shown that 5,6-diHETrE and 8,9-diHETrE can produce dose-dependent vasodilatation by modulating the bioavailability of NO via endothelial NO synthase (519). In a lipidomics study of 16 healthy male volunteers, 5,6-diHETrE and 8,9-diHETrE levels were elevated after treatment with the non-steroidal anti-inflammatory drug ibuprofen (518). In another study of overweight and obese men, the level of 5,6-diHETrE was increased after intervention with diclofenac (520). 11,12-diHETrE was shown to relax U-46619-contracted artery rings (521) and was implicated the candidate biomarker for the noninvasive diagnosis of NASH (507). 14,15-diHETrE has been documented to be elevated in pregnancy-induced hypertension (522). In the present study, we found that treatment of ALA and SDA significantly increased the concentration of 5,6-diHETrE by 1.3-fold and 2.1-fold, respectively (Figure 29H). DHA treatment significantly increased 8,9-diHETrE concentration by 2.3-fold (Figure 29I). EPA treatment significantly increased the levels of 5,6-, 8,9-, 11,12-, and 14,15-diHETrEs by 4.7-fold, 5.5-fold, 3.4-fold, and 5.1-fold, respectively (Figure 29H-K). This results suggest that EPA has a particular effect on the production of diHETrEs.

As the precursor for all PGs, TXs, and LTs, AA production in H19-7 cells was also significantly affected by ω-3 fatty acids. As shown in Figure 30A, treatment of ALA, EPA, and

SDA significantly increased the concentration of AA by 9.7-fold, 7.1-fold, and 6.9-fold, respectively. Similarly, the level of adrenic acid (AdA), derived directly from AA, was also significantly increased by ALA (4.8-fold) and EPA (10.9), respectively (Figure 30B). AdA is one of the most abundant fatty acids in the early human brain (523). AdA was found to function as endogenous endothelium- and zona glomerulosa-derived hyperpolarizing factors in the adrenal cortex and contribute to the regulation of adrenal blood flow (524). These results may explain why ALA is the least anti-inflammatory and DHA is the most anti-inflammatory among ω-3 fatty acids.

5.4.9 Mead acid-derived 5-HETrE production in response to ω-3 fatty acids

5-HETrE is produced by the action of 5-LOX when mead acid (ω-9) is the substrate. In the present study, as shown in Figure 31, 5(S)-HETrE was not detected in untreated cells; however, all ω-3 fatty acids dramatically increased its level. 5-HETrE can convert to LTA3 via the 5-LOX-leukotriene pathway (525). 5-HETrE has also been found to depress osteoblast activity (526). In addition, 5-HETrE has a potentiating effect on human platelet aggregation (527).

5.4.10 Nitrated unsaturated fatty acids production in response to ω-3 fatty acids

9- and 10-nitrooleates serve as potent endogenous signaling mediators as ligands for PPARγ (528), and may be useful for treating diseases associated with dysregulated immune homeostasis, such as inflammatory bowel disease (529), atherosclerosis (530), LPS-induced acute lung injury (531), allergic airway disease (532), hypertension (533, 534), cisplatin nephropathy (535), and atrial fibrillation (536). A recent study has shown that treatment of 10-nitro-oleic acid downregulated expression and activity of the inflammatory transcription factor NF-κB while upregulating those of PPARγ (537). In the present study, as shown in Figure 32A, the production of 9-nitrooleates was dramatically inhibited by ALA to “not detectable”. While, the level of 10-

nitrooleates was only detectable in DHA- and SDA-treated cells (Figure 32B). These results suggest again the role of ω-3 fatty acids in chronic inflammatory diseases.

5.4.11 Non-detectable eicosanoids

Metabolites included in the complete eicosanoid panel but were not detected in our samples are listed in Table 3.

5.5 Conclusions

All the metabolites that have been detected in our samples are summarized in the pathways as shown in Figure 33. Metabolites with significant ($p<0.05$) changes in H19-7 cells upon treatment of different ω-3 fatty acids are summarized in Figure 34(A-D). Fold changes were calculated by the concentration of each metabolite divided by corresponding control concentration. A fold change = 0 means the metabolite was not affected by the treatment; > 0 means the production of that metabolite was significantly increased upon the treatment; and < 0 means the production of that metabolite was significantly decreased upon the treatment. The anti- or pro-inflammatory properties of each metabolite are shown in different color. Green means anti-inflammatory, red means pro-inflammatory, and yellow means both properties. However, many metabolites do not have a documented conclusion of anti- or pro-inflammatory property, in which case, we temporarily mark them based on the existing literature and their precursors, since ω-3 metabolites are usually anti-inflammatory but ω-6 are usually pro-inflammatory. A Venn diagram was further drawn to summarize the metabolites that were significantly ($p<0.05$) affected upon each treatment of ω-3 fatty acid in H19-7 cells (Figure 35).

Based on our lipidomics results, we may conclude that: 1) ω-3 fatty acids affect the production of eicosanoids by all the pathways, including LOX, COX, CYP, and non-enzymatic

autoxidation pathways. 2) ω-3 fatty acids are natural anti-inflammatory compounds that can convert to a lot of anti-inflammatory eicosanoids, such as PGD3, PGE3, 14-HDoHE (precursor for maresin1), and 17-HDoHE (precursor for resolvin D and protectin D) under normal conditions. 3) Production of many pro-inflammatory metabolites, such as PG 2-series, are also increased by ω-3 fatty acids, especially by EPA; 3) treatment of ω-3 fatty acids, mainly DHA can increase the ROS production, such as HDoHEs in neurons. 4) The effects on the production of eicosanoids are consistent among all four ω-3 fatty acids. They always change the levels of eicosanoids in the same direction. 5) In general, the efficacy of anti-inflammatory potent is DHA&EPA > SDA >> ALA. ALA seems having much less potent when compared with the others as evidenced by less effects on eicosanoid production (Figure 34A). As shown in Figure 34 and Figure 35, the pattern of eicosanoids by SDA treatment is most close to the pattern by EPA, which confirmed the proposal that SDA is the surrogate for EPA. The pattern of DHA is also very similar to EPA and SDA except that DHA can uniquely affect the production of some DHA-derived metabolites. 6) Compared to EPA, SDA is much less potent in increasing the production of anti-inflammatory metabolites, but also much less potent in increasing the production of pro-inflammatory metabolites, indicating SDA may have advantages over EPA. 7) In addition to being a surrogate for EPA, SDA has its unique roles in the biosynthesis of eicosanoids, such as decreased PGD1 level, suggesting that SDA may have its own specific biological functions different from DHA and EPA. Finally, this lipidomics study of ω-3 fatty acids is valuable and provides lots of inspirations for future studies, especially in the area of chronic inflammatory diseases, such as AD, obesity, diabetes, arthritis, skin disorders, and tumors, as well as in the area of cardiovascular system where ω-3 fatty acids act as vasodilator, such as platelet aggregation, muscle contraction, intraocular and

blood pressure, and the area of host immune defense, such as influenza, would healing, asthma, and allergy.

Table 3 Metabolites included in the complete eicosanoid panel but are not detected in our samples

6k PGF1α	19oh PGF2a	TXB1	6R-LXA4	5,6-diHETE
PGE2	20oh PGF2a	TXB3	6S-LXA4	8,15-diHETE
PGD2	19oh PGE2	2,3 dinor TXB2	15R-LXA4	5-oxoETE
11b PGF2a	20oh PGE2	11d-TXB2	LXA5	12-oxoETE
PGF1a	2,3 dinor 11b PGF2a	20oh LTB4	LXB4	15-oxoETE
PGE1	PGFM	20cooh LTB4	HXA3	13-oxoODE
d17 6k PGF1a	PGEM	6t LTB4	Resolvin E1	15 oxoEDE
PGF3a	11b PGE2	12epi LTB4	Resolvin D1	20-HETE
dihomo PGF2a	PGK2	6t,12epi LTB4	Protectin D1	19(R)-HETE
dihomo PGE2	15d PGA2	12oxo LTB4	15t-Protectin D1	18(S) HEPE
dihomo PGD2	15d PGD2	LTC4	10S-Protectin D1	5,6-EET
dihomo PGJ2	15d PGJ2	LTD4	Maresin-1	8,9-EET
dihomo 15d PGD2	5-iso PGF2a VI	LTE4	Resolvin D2	11,12-EET
6k PGE1	8-iso PGF2a III	11t LTC4	Resolvin D3	14,15-EET
6,15 dk-,dh- PGF1a	2,3 dinor 8-iso PGF2a	11t LTD4	Resolvin D5	14(15) EpETE
15k PGF1a	2,3 dinor-6k PGF1a	11t LTE4	dhk PGF2a	17(18) EpETE
15k PGF2a	PGK1	14,15 LTC4	dhk PGE2	16(17) EpDPE
15k PGE2	8-iso PGF3a	14,15 LTD4	bicyclo PGE2	19(20) EpDPE
15k PGD2	8-iso-15k PGF2b	14,15 LTE4	11b dhk PGF2a	9,10 EpOME
dh PGF2a	tetranor-PGDM			12,13 EpOME

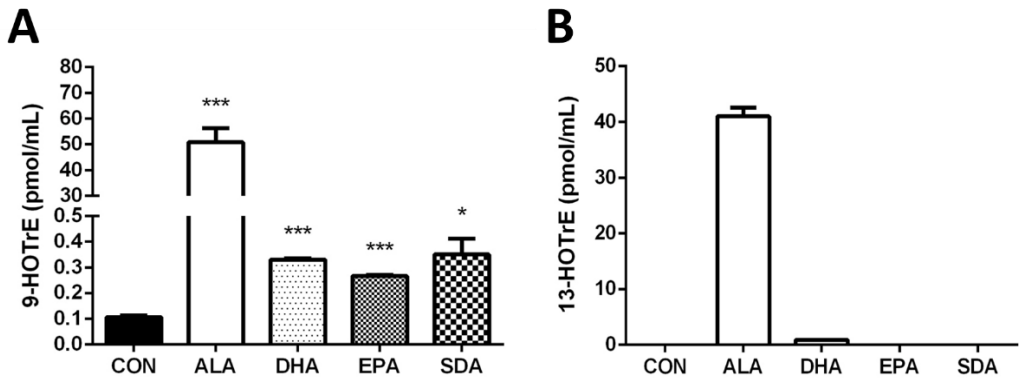

Figure 17 Changes of ALA-derived eicosanoids by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 9-HOTrE **(A)** and 13-HOTrE **(B)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

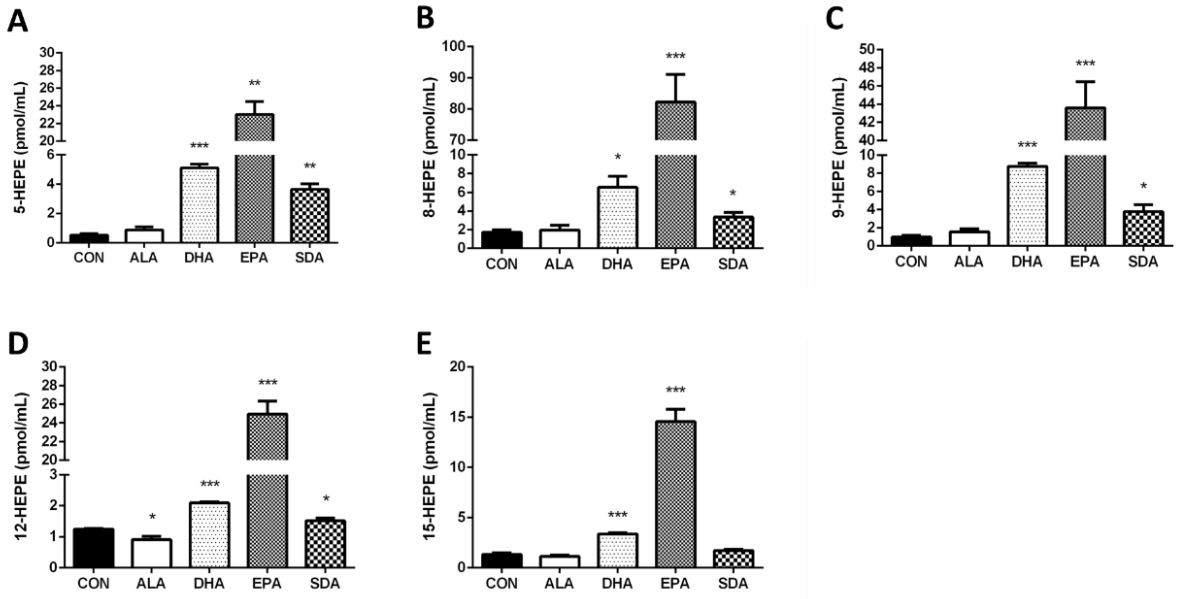

Figure 18 Changes of EPA-derived eicosanoids by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 5-HEPE **(A)**, 8-HEPE **(B)**, 9-HEPE **(C)**, 12-HEPE **(D)**, and 15-HEPE **(E)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

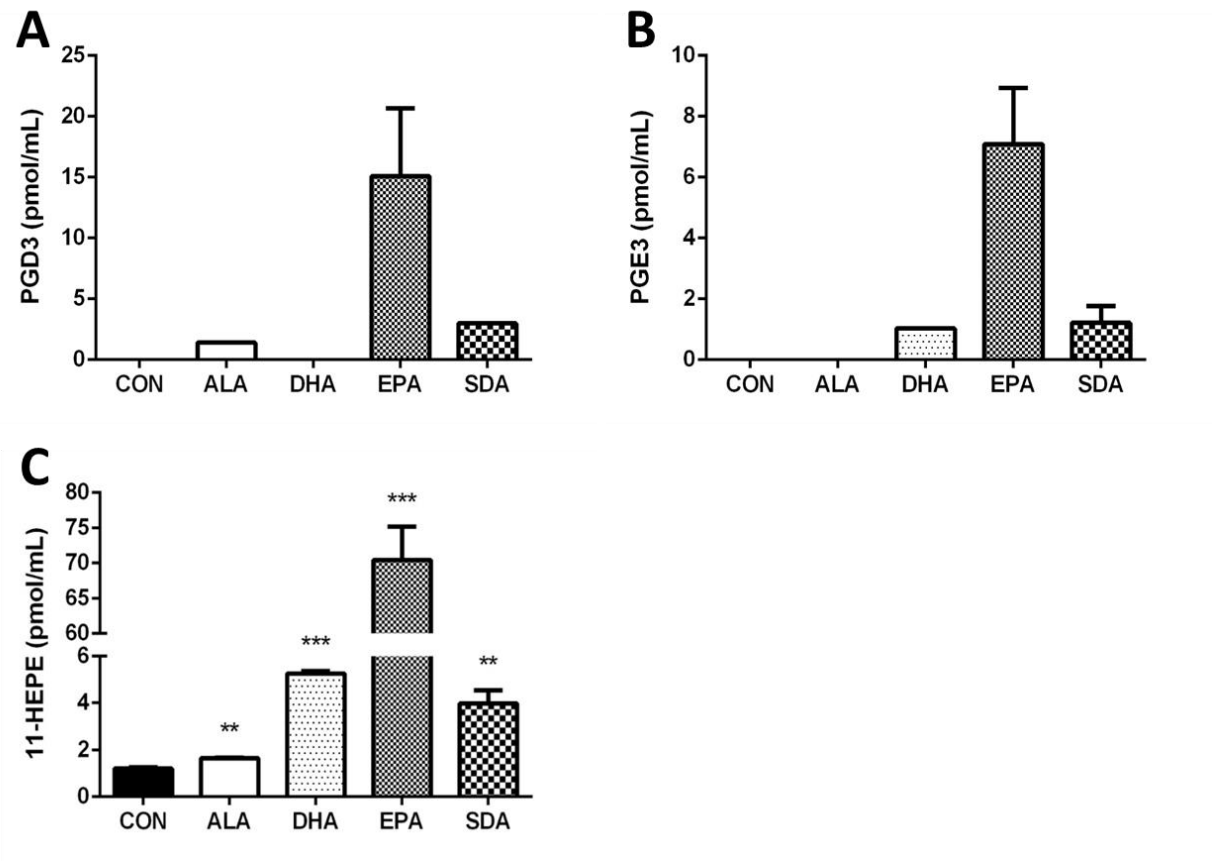

Figure 19 Changes of EPA-derived eicosanoids by COX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. PGD3 **(A)**, PGE3 **(B)**, and 11-HEPE **(C)**, concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

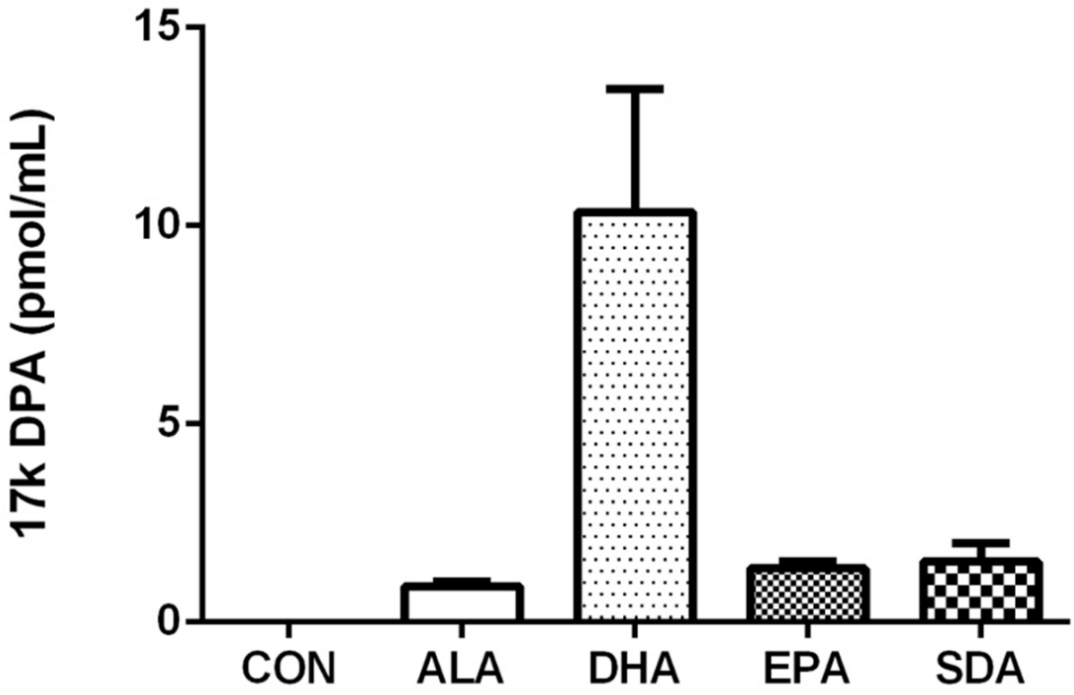

Figure 20 Changes of DPA-derived 17k DPA by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 17k DPA concentration (pmol/mL) in cell culture supernatant was measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM.

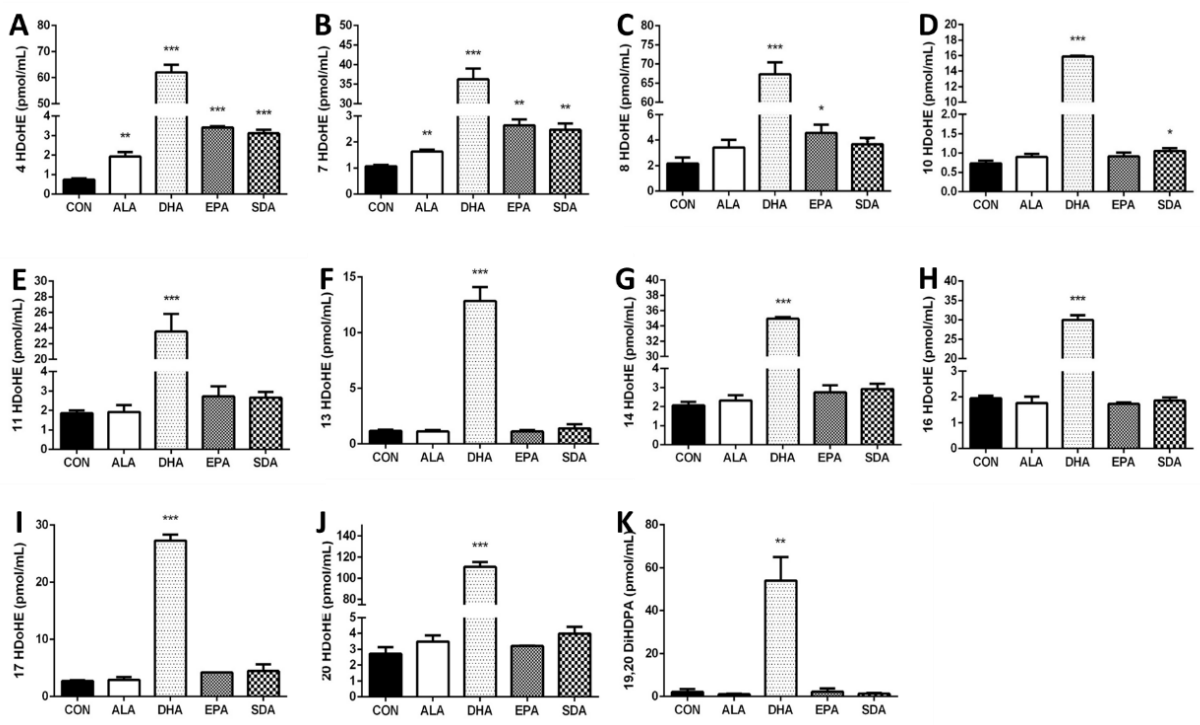

Figure 21 Changes of DHA-derived eicosanoids by non-enzymatic autoxidation pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 4HDoHE **(A)**, 7HDoHE **(B)**, 8HDoHE **(C)**, 10HDoHE **(D)**, 11HDoHE **(E)**, 13HDoHE **(F)**, 14HDoHE **(G)**, 16HDoHE **(H)**, 17HDoHE **(I)**, 20HDoHE **(J)**, and 19,20DiHDPA **(K)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

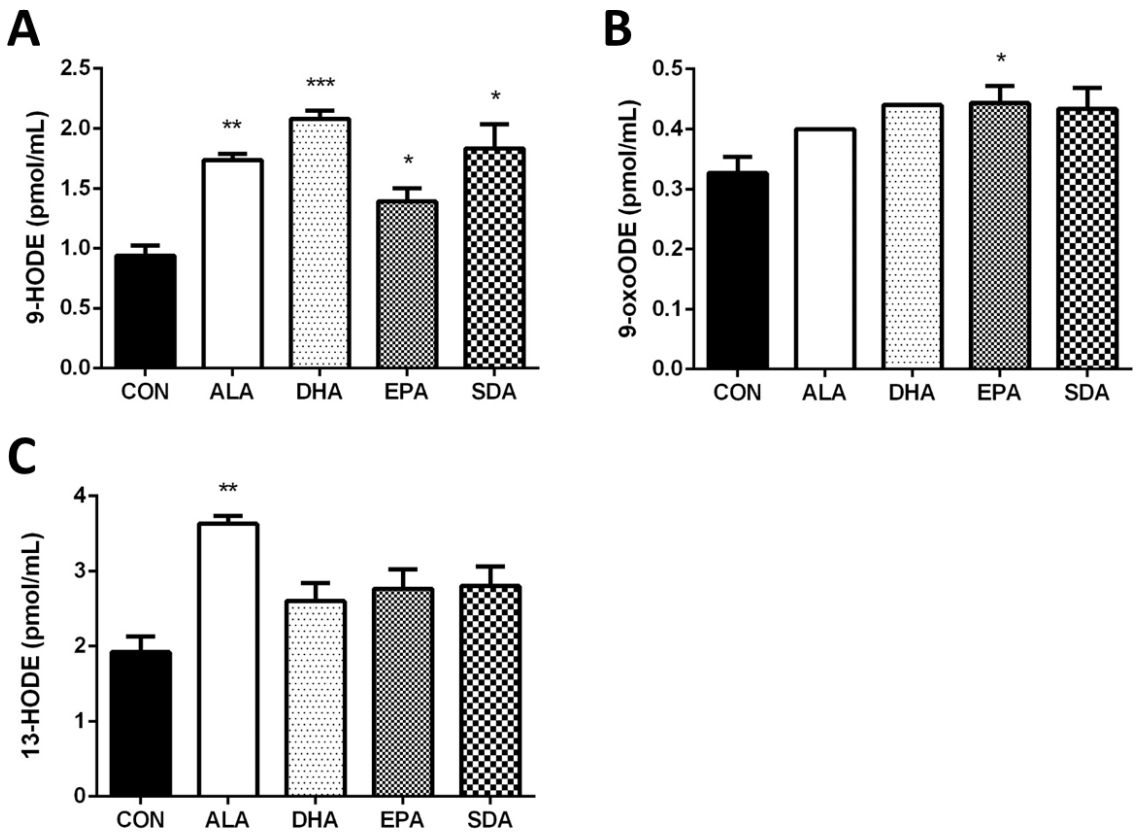

Figure 22 Changes of LA-derived eicosanoids by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 9-HODE **(A)**, 9-oxoODE **(B)**, and 13-HODE **(C)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

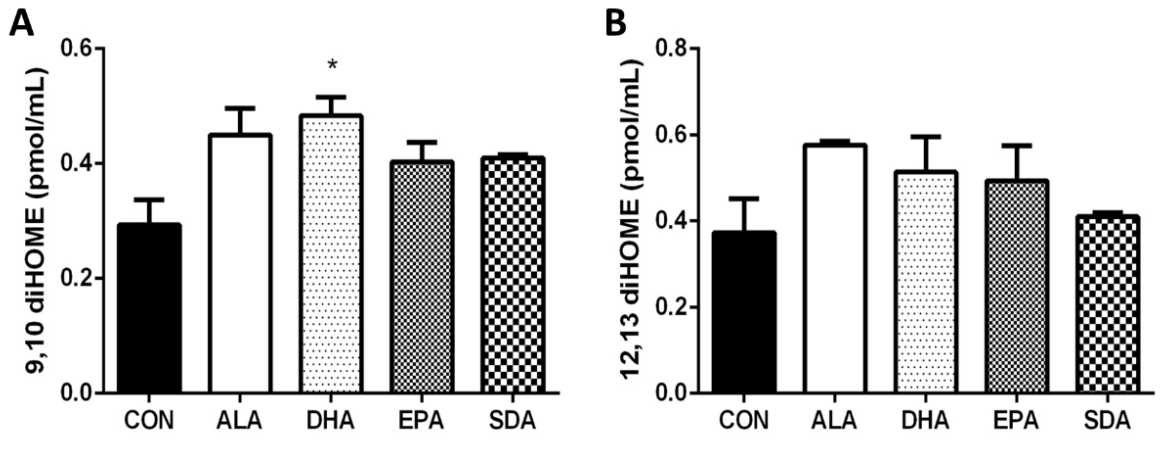

Figure 23 Changes of LA-derived eicosanoids by CYP pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 9,10-diHOME **(A)** and 12,13-diHOME **(B)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, different from BSA-ethanol vehicle treated control cells.

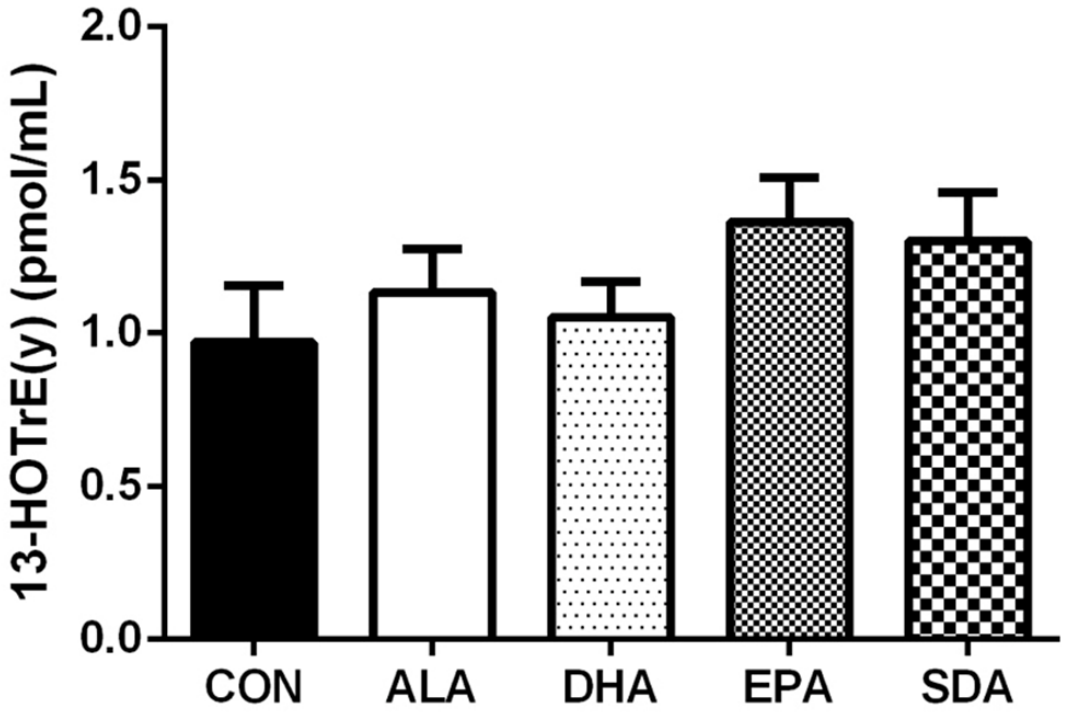

Figure 24 Changes of GLA-derived 13-HOTrE(γ) by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 13-HOTrE(γ) concentration (pmol/mL) in cell culture supernatant was measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM.

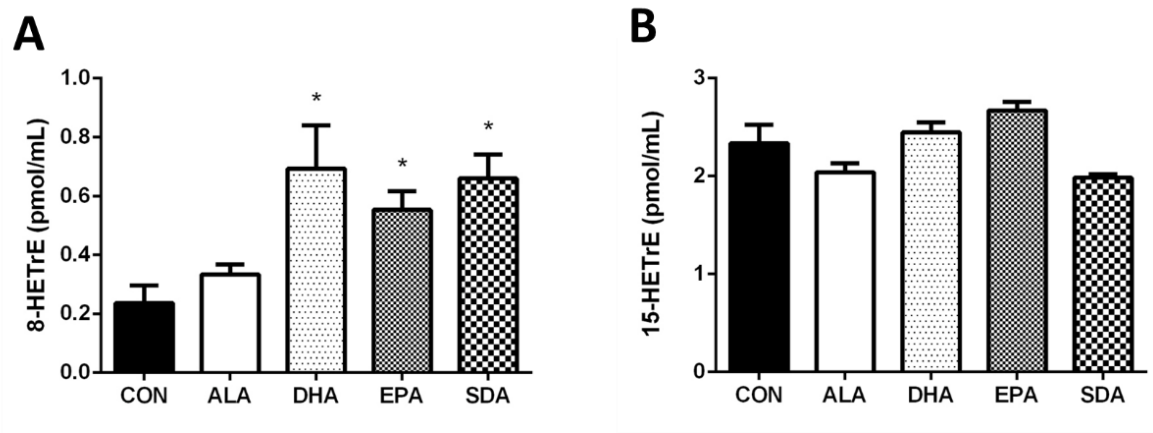

Figure 25 Changes of DGLA-derived eicosanoids by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 8-HETrE **(A)** and 15-HETrE **(B)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, different from BSA-ethanol vehicle treated control cells.

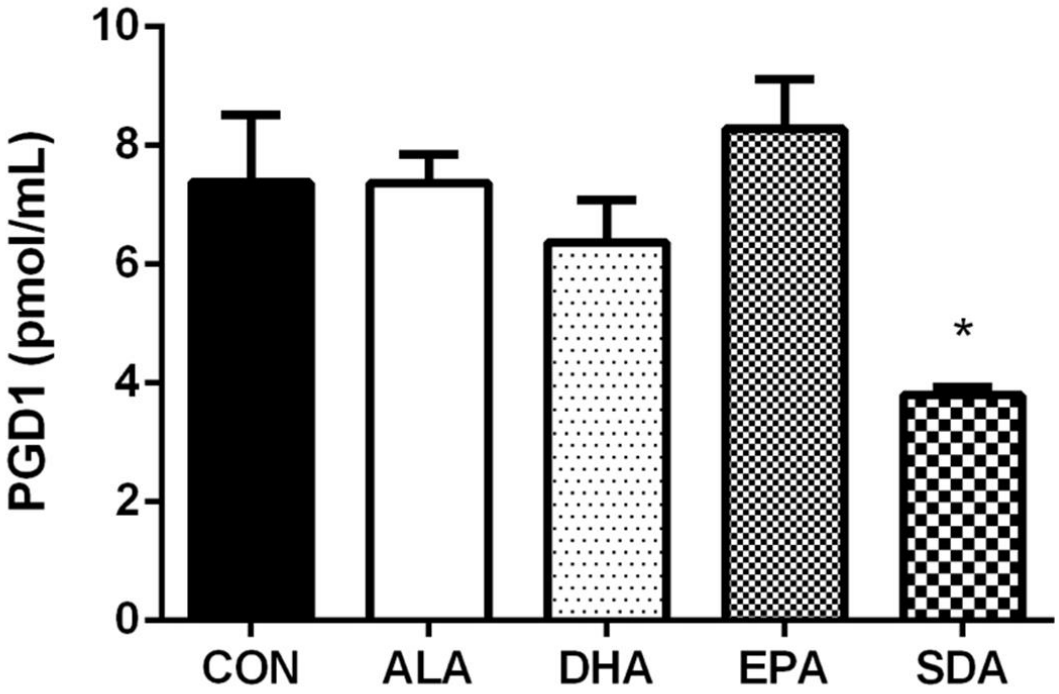

Figure 26 Changes of DGLA-derived PGD1 by COX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. PGD1 concentration (pmol/mL) in cell culture supernatant was measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, different from BSA-ethanol vehicle treated control cells.

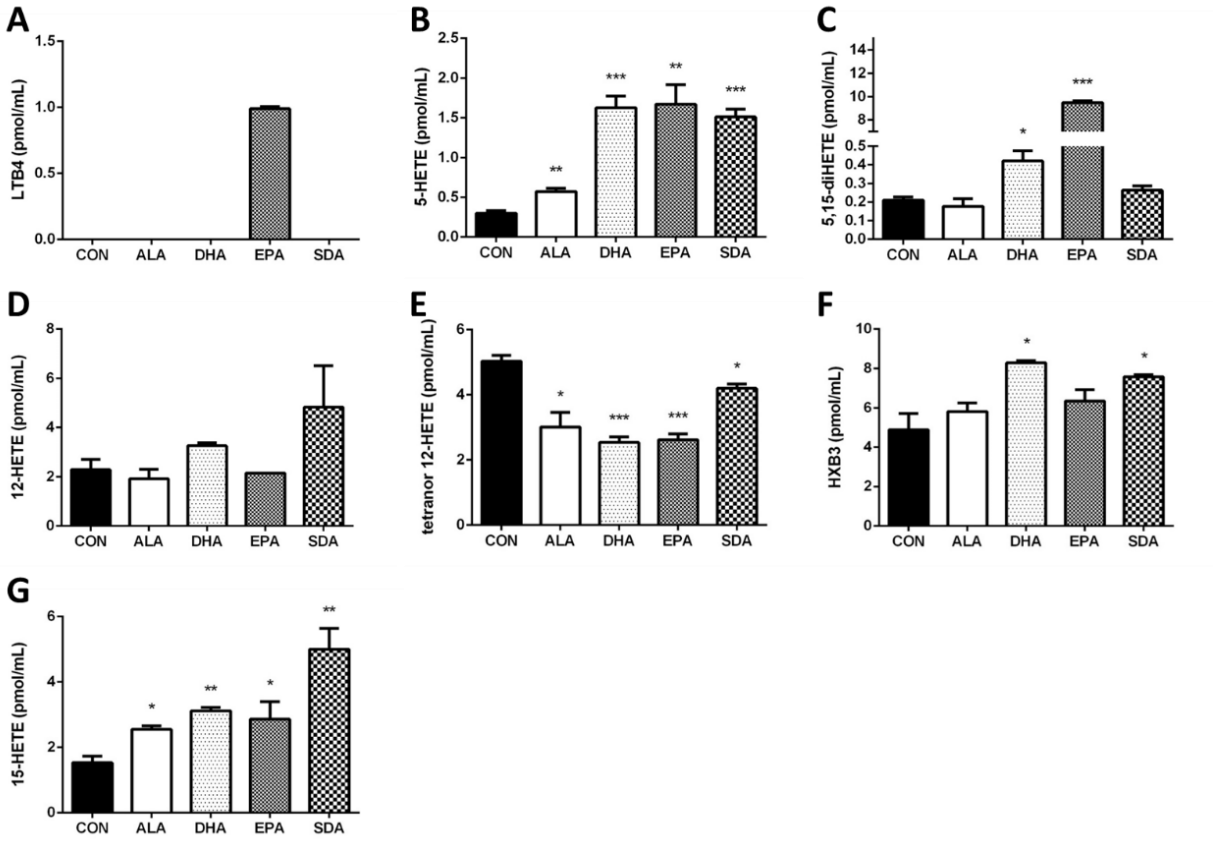

Figure 27 Changes of AA-derived eicosanoids by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. LTB4 **(A)**, 5-HETE **(B)**, 5,15-diHETE **(C)**, 12-HETE **(D)**, tetranor 12-HETE **(E)**, HXB3 **(F)**, and 15-HETE **(G)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

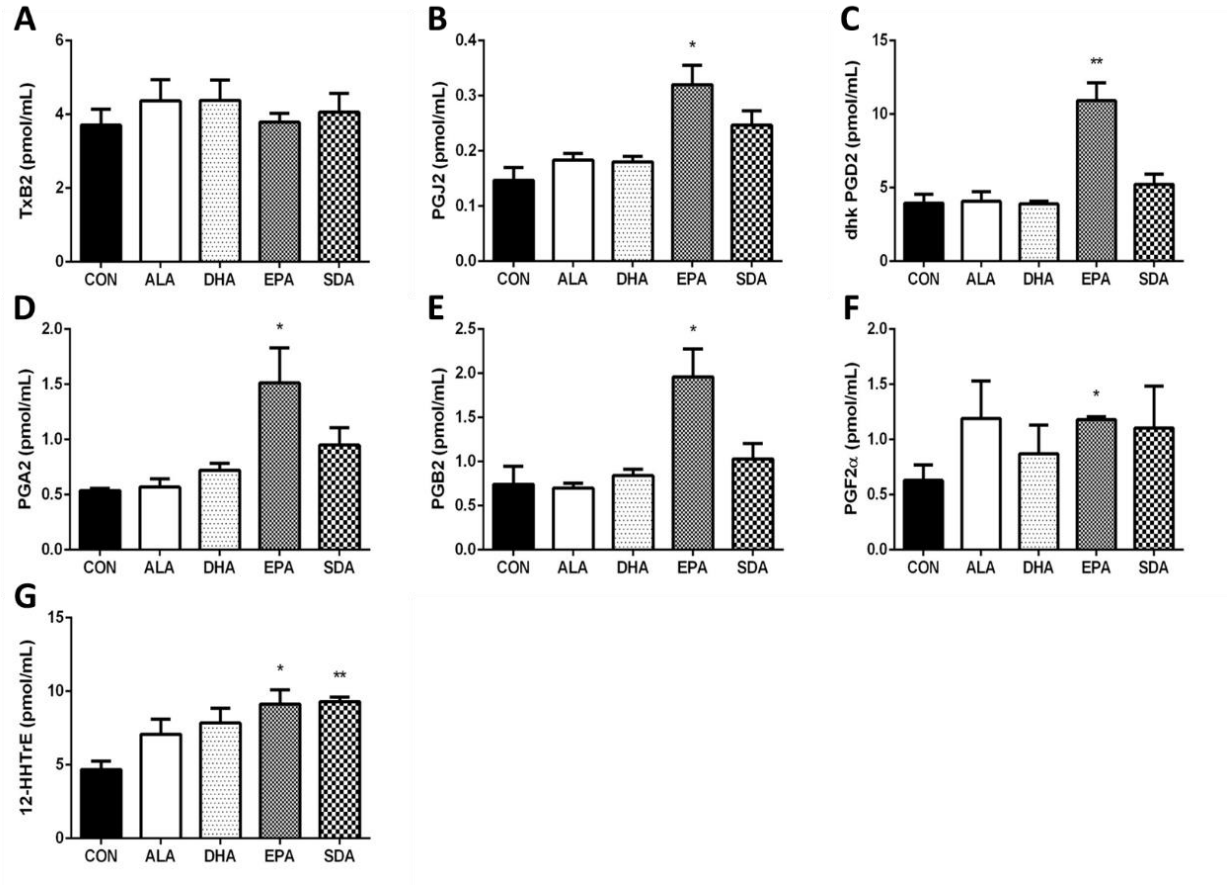

Figure 28 Changes of AA-derived eicosanoids by COX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. TXB2 **(A)**, PGJ2 **(B)**, dhk PGD2 **(C)**, PGA2 **(D)**, PGB2 **(E)**, PGF2α **(F)**, and 12-HHTrE **(G)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean$\pm$SEM. *P < 0.05, **P < 0.01, different from BSA-ethanol vehicle treated control cells.

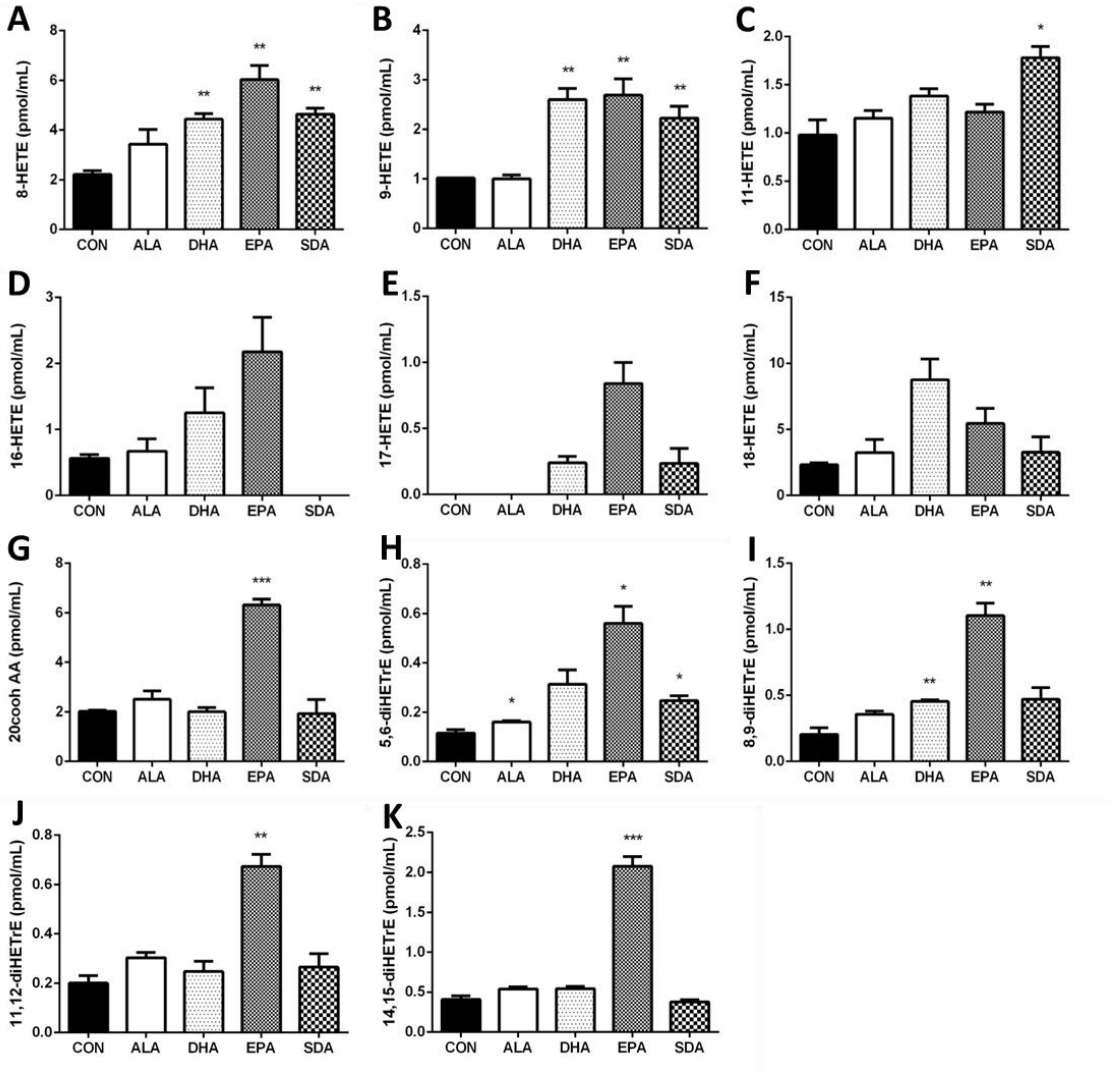

Figure 29 Changes of AA-derived eicosanoids by CYP pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 8-HETE **(A)**, 9-HETE **(B)**, 11-HETE **(C)**, 16-HETE **(D)**, 17-HETE **(E)**, 18-HETE **(F)**, 20-COOH-AA **(G)**, 5,6-diHETrE **(H)**, 8,9-diHETrE **(I)**, 11,12-diHETrE **(J)**, and 14,15-diHETrE **(K)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

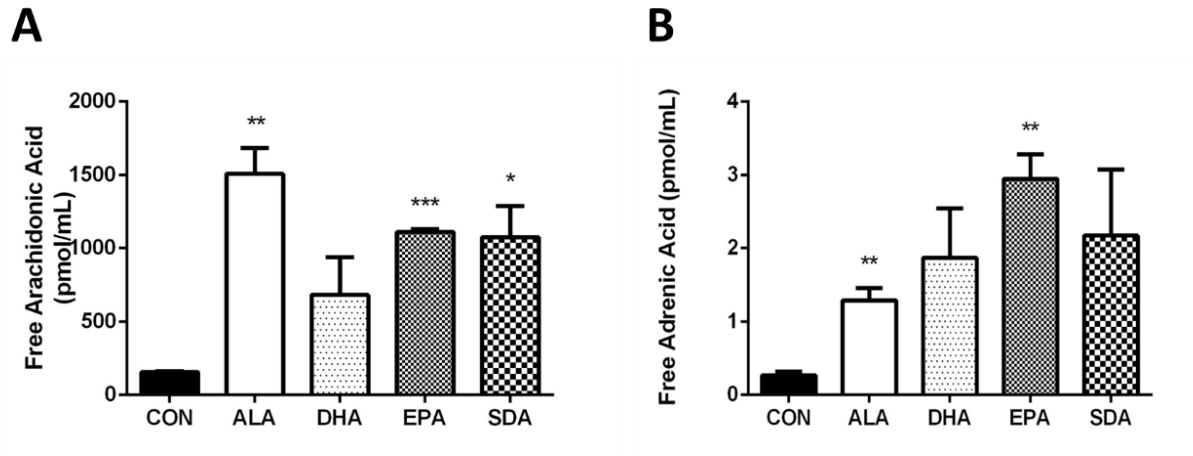

Figure 30 Changes of AA and AdA in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. AA **(A)** and AdA **(B)** concentrations (pmol/mL) in cell culture supernatant were measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, different from BSA-ethanol vehicle treated control cells.

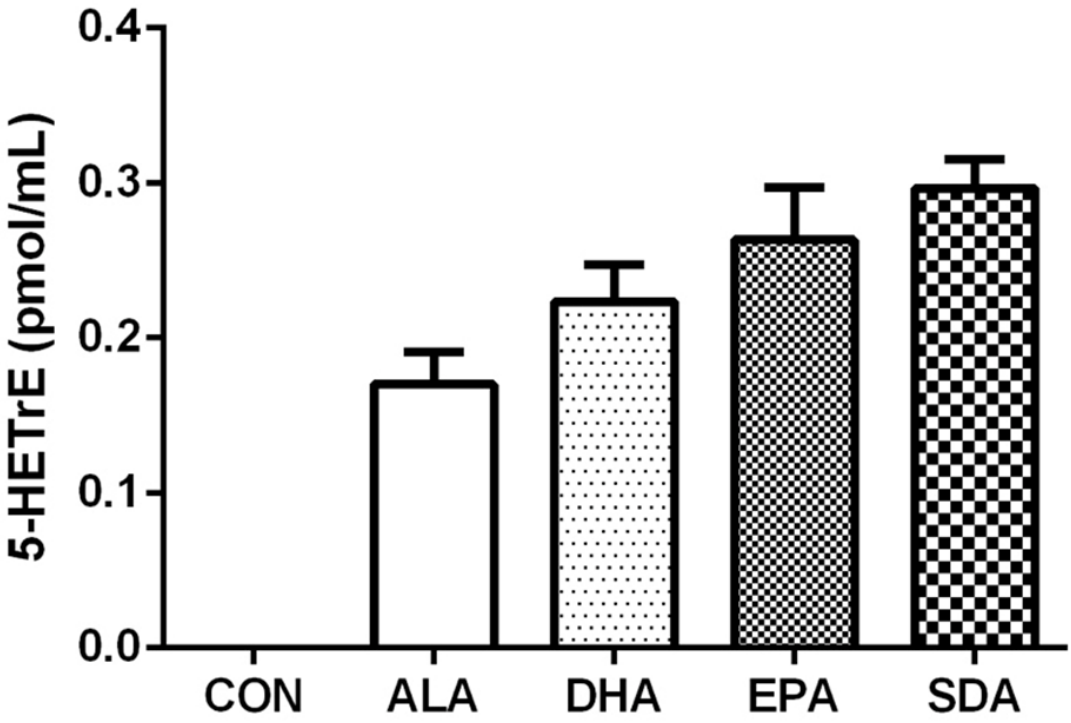

Figure 31 Changes of mead acid-derived 5-HETrE by LOX pathway in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 5-HETrE concentration (pmol/mL) in cell culture supernatant was measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM.

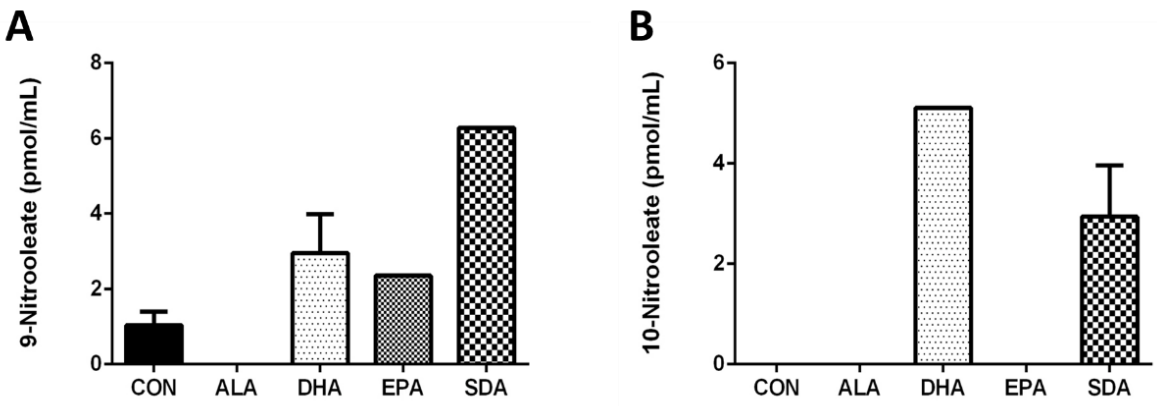

Figure 32 Changes of nitrooleates in H19-7 cells upon treatment of ω-3 fatty acids.

H19-7 hippocampal cells were incubated with 100 μM ALA, DHA, EPA, SDA, or BSA-ethanol vehicle for 48 hours. 9-nitrooleate **(A)** and 10-nitrooleate **(B)** concentrations (pmol/mL) in cell culture supernatant was measured by lipidomics. Values were obtained from three independent experiments and are expressed as the mean±SEM.

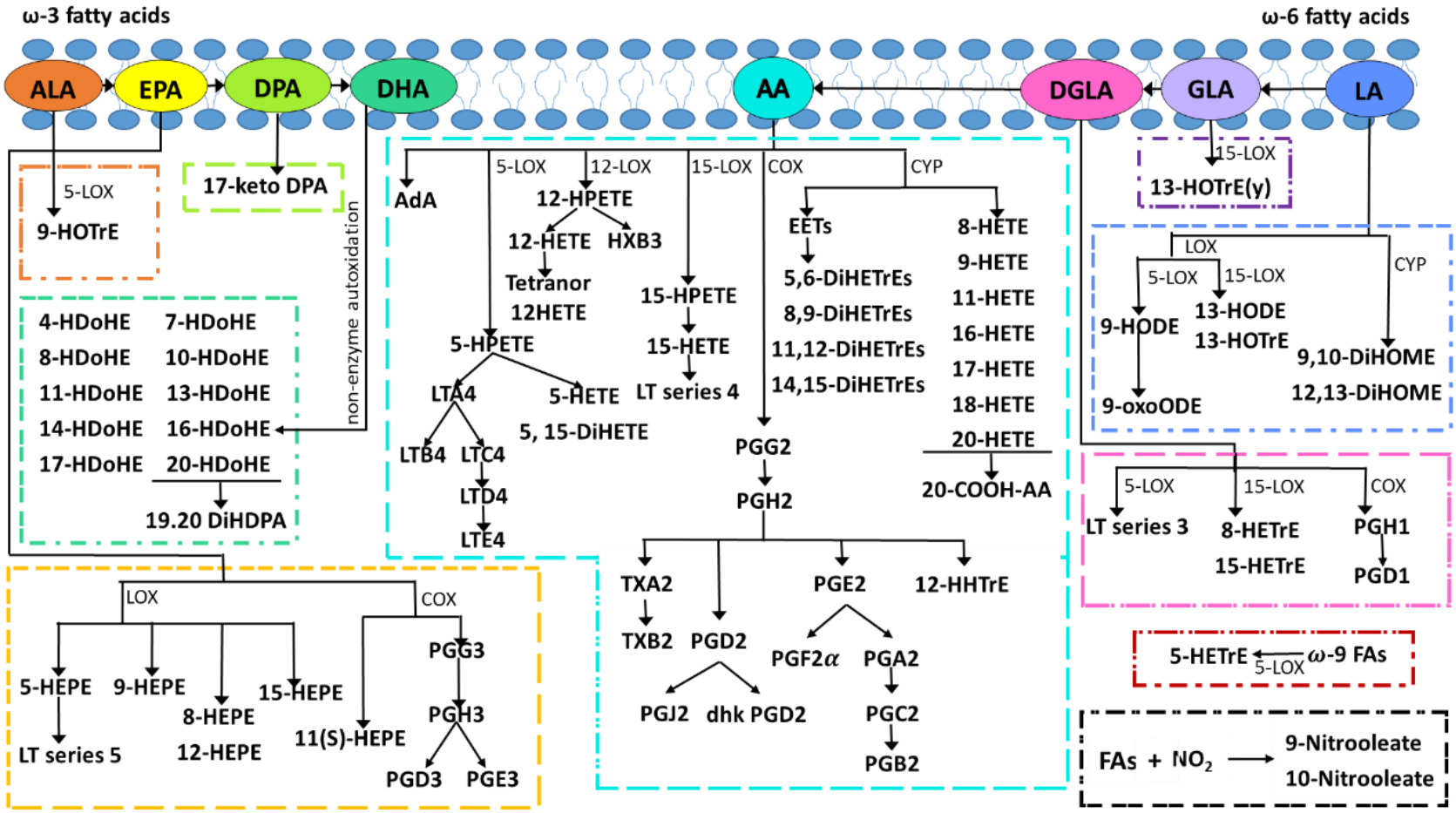

Figure 33 Metabolites detected in H19-7 cells in pathways.

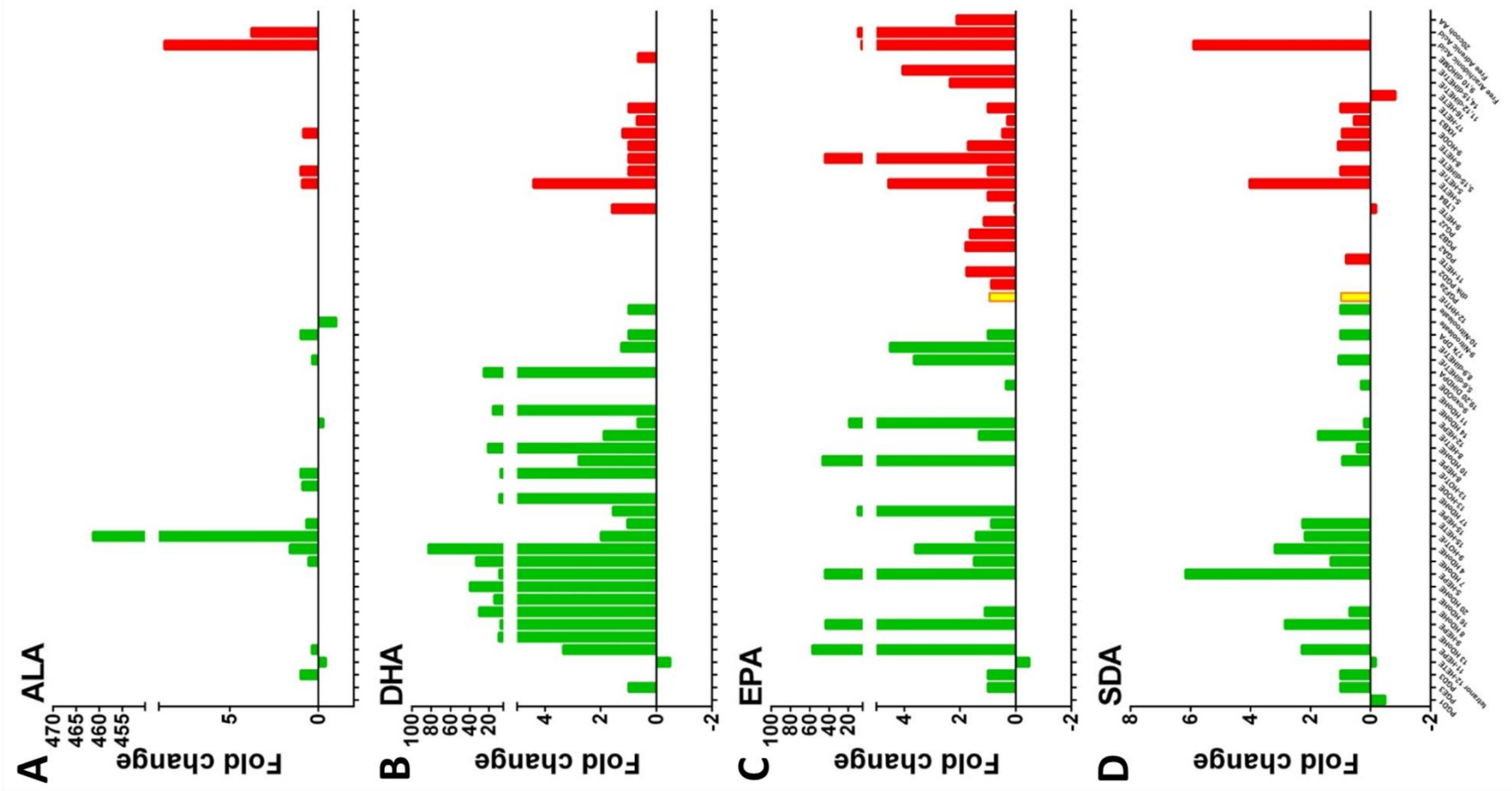

Figure 34 Significant (p<0.05) changes of all the metabolites in H19-7 cells upon treatment of ω-3 fatty acids.

ALA **(A)**, DHA **(B)**, EPA **(C)**, and SDA **(D)**. Fold change was calculated by the concentration of each metabolite divided by corresponding control concentration. Metabolites with anti-inflammatory properties are shown in Green, with pro-inflammatory properties are shown in Red. The one in Yellow shows both properties.

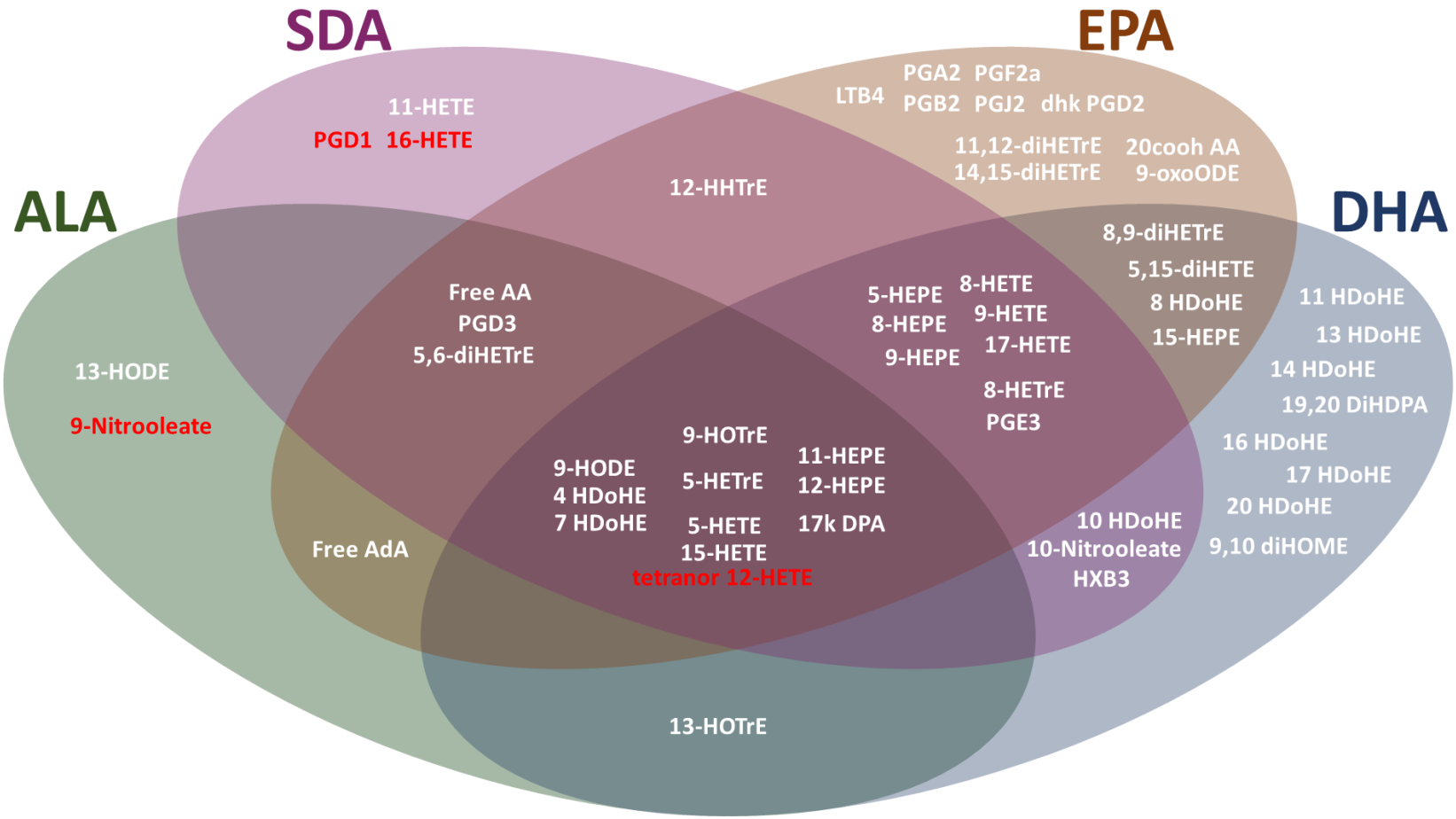

Figure 35 Venn diagram summarizing the metabolites that were significantly ($p<0.05$) affected upon each treatment of ω-3 fatty acid in H19-7 cells.

Color white means the production of the metabolite was increased and color red means the production of the metabolite was decreased.

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