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I am submitting herewith a dissertation written by Robert Clements Gillis entitled "Effects of n-3 and n-6 Polyunsaturated Fatty Acids on Human Promyelocytic HL-60 Cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Michael D. Karlstad
Major Professor

We have read this dissertation
and recommend its acceptance:

Brian J. Daley

Patricia K. Tithof

Jay Whelan

Accepted for the Council:

Anne Mayhew
Vice Provost and Dean of
Graduate Students

(Original signatures are on file with official student records)

Effects of n-3 and n-6 Polyunsaturated Fatty Acids on Human Promyelocytic HL-60 Cells

A Dissertation
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Dedication

This dissertation is dedicated to my wife, Heidi Gillis; my parents, David and Katherine Gillis; my brother James Gillis; and my sister, Kathleen Swartz for always believing in me and encouraging me to reach higher in order to achieve my goals.

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Abstract

Enteral nutrition with eicosapentaenoic acid (EPA; 20:5 n-3) and γ-linolenic acid (GLA; 18:3 n-6) decreased pulmonary inflammation by reducing neutrophil counts and chemotactic factors in bronchoalveolar lavage fluid during acute respiratory distress syndrome (ARDS). We hypothesize that the anti-inflammatory effects of EPA and GLA may be due, in part, to induction of neutrophil apoptosis. Neutrophil apoptosis is an important physiological process in the resolution of pulmonary inflammation. Furthermore, pro-inflammatory eicosanoids formed from arachidonic acid (AA) by lipoxygenase (LO) and cyclooxygenase (COX) pathways have been shown to inhibit apoptosis in certain cell types. The purpose of this dissertation is to determine whether EPA and GLA, alone or in combination, trigger apoptotic cell death in the human promyelocytic leukemia HL-60 cell line. This dissertation will also determine whether inhibition of LO and COX increases apoptosis with AA, the combination of EPA and GLA, and EPA in HL-60 cells, *in vitro*. Finally, this dissertation will determine the apoptotic gene profile of EPA/GLA-treated HL-60 cells.

Study 1, HL-60 cells were incubated with 10, 20, 50, and 100 μM EPA, GLA or various combinations of EPA and GLA for 2, 4, 8, 12 and 24 hours. Oleic acid (18:1 n-9) was used as a fatty acid control. Flow cytometry using dual-staining with propidium iodide and annexin V-FITC assessed apoptosis, necrosis and viability. Apoptosis was verified by DNA fragmentation as assessed by agarose gel electrophoresis. EPA, GLA, and various combinations of EPA and GLA significantly induced apoptosis and reduced cell viability in HL-60 cells.

Viability was significantly reduced to the same extent with the combination of 50 μM EPA\20 μM GLA as compared with 100 μM EPA. These data indicate that EPA and GLA, alone or in combination, reduce cell survival by induction of apoptosis. Thus, induction of apoptosis by select dietary n-3 (EPA) and n-6 (GLA) polyunsaturated fatty acids may be the mechanism of reduces pulmonary inflammation in ARDS.

Study 2 was conducted as follows: HL-60 cells were incubated with 50 μM AA and an enzyme inhibitor (1-10 μM) for either COX, LO, 12-LO, and 5-LO for 12 hours. Flow cytometry was used to assess viability, apoptosis, and necrosis. Apoptosis was further assessed using TUNEL and DNA fragmentation. The highest concentration of LO inhibitors, but not COX inhibitors, decreased viability and increased apoptosis and necrosis in the presence of exogenous AA. These results suggest that disruption of the metabolism of AA by LO, in particular 5-LO, decreases cell survival and increases apoptosis. Thus, downstream metabolic processing of AA by LO but not COX plays a critical role in the regulation of HL-60 cell apoptosis.

Study 3 was conducted as follows: HL-60 cells were incubated with 50 μM EPA /20 μM GLA in the presence of an enzyme inhibitor (1-10 μM) for 12 hours. Compounds were used to inhibit COX (ibuprofen), 12-LO (baicalein), or 5-LO (AA-861). Flow cytometry assessed viability, apoptosis, and necrosis. 5-LO inhibition reduced cell viability and increased cell death (apoptosis+necrosis) in EPA/GLA-treated HL-60 cells. Inhibition of COX 1 and 2 and 12-LO had no significant effect on cellular viability and death in EPA/GLA-treated HL-60 cells.

Adding leukotriene B_4 counteracted the effect of 5-LO inhibition on apoptosis in EPA/GLA-treated HL-60 cells. These data suggest that the processing of EPA and GLA by 5-LO is critical to HL-60 cell survival.

Study 4 was conducted as follows: HL-60 cells were incubated with 50 μM EPA in the presence of an enzyme inhibitor (1-10 μM) for 12 hours. Compounds were used to inhibit either COX 1 and 2 (ibuprofen), 5-, 12-, 15-LO (NDGA), 12-LO (baicalein), 5-LO (AA-861), and 5-LO activating protein (MK-886). Eicosanoid (0.001-1.0 μM) add back experiments were also conducted; LTB_4 and 5-HETE with 5-LO inhibition and 12-HETE with 12-LO inhibition. Flow cytometry, TUNEL assay, and caspase-3 western blotting were used to assess apoptosis. Inhibition of COX 1 and 2 had no effect on apoptosis. Inhibition of 5-LO and 12-LO significantly increased apoptosis in EPA-treated HL-60 cells. Furthermore, addition of LTB_4 reduced apoptosis to levels significantly lower than EPA-treated HL-60 cells alone. 5-HETE and 12-HETE also lowered apoptosis to control levels. These data indicate that inhibition of LO, particularly 5-LO, increased apoptosis in EPA-treated HL-60 cells. Furthermore, this study clearly demonstrated that the products of the LO enzymes, particularly LTB_4, are critical in the regulation of apoptosis in EPA-treated HL-60 cells.

Study 5 was conducted as follows: HL-60 cells were incubated with 50 μM EPA and 20 μM GLA for 12 hours. Apoptotic gene profiles were assessed using GEArray original series human apoptosis-2 gene arrays. Total RNA was extracted and probes were synthesized with a primer mix unique for each array. Arrays were hybridized and developed in a non-isotopic manner. Arrays were

visualized using a 16-bit CCD camera. The apoptotic gene profile for no treatment and EPA/GLA-treated HL-60 cells after 12-hours differed from each other. When comparing density values as a percent of control, all genes assayed were down-regulated. When comparing arrays' relative intensities, several genes were different between control and treatment groups. For example, Bad, Bax, and Fas were down regulated and Bax, Bcl-2, and p53 were up regulated as compared with control.

This dissertation has provided a possible mechanism for the observed effect of EPA and GLA in human and animal studies of acute respiratory distress syndrome (ARDS): i.e., that enteral nutrition with specialized diets containing EPA and GLA reduced the number of neutrophils and reduced the levels of LTB_4 in the bronchoalveolar lavage fluid, reduced pulmonary inflammation, and improved clinical outcomes in patients and animals with ARDS. This dissertation demonstrates that the observed clinical results may be due to increased neutrophil apoptosis induced by EPA and GLA. Induction of apoptosis likely occurs through a change in the eicosanoid profile as well a genetic change through the up-regulation and down-regulation of various pro- and anti-apoptotic genes. Thus, the ability to alter the inflammatory cell phospholipids profile to reduce the amount of AA available for metabolism to pro-inflammatory eicosanoids, in conjunction with 5-lipoxygenase inhibitory compounds, may lead to new therapies that will increase neutrophil apoptosis and provide a injury-limiting pathway in the resolution of pulmonary inflammation in ARDS.

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Part 1

Introduction

I. Introduction

There is strong evidence demonstrating that polyunsaturated fatty acids (PUFA) from the n-3 series, as well as the combination of n-3 and n-6 series PUFA, are beneficial in a variety of health problems such as cardiovascular disease, arthritis, cancer, and pulmonary inflammation [1-4]. Certain dietary PUFA have the ability to induce apoptosis in a variety of cell types. The mechanism by which n-3 and n-6 PUFA induce beneficial effects may be through induction of apoptosis of the effector cell, i.e. neutrophils in acute respiratory distress syndrome.

Essential fatty acids are precursors of the eicosanoids, which play a critical role in inflammatory cell function. Diets rich in arachidonic acid (AA, 20:4 n-6) lead to the formation of pro-inflammatory biological compounds. These pro-inflammatory eicosanoids play an essential role in the development of septic shock, systemic inflammatory response syndrome, acute lung injury, and acute respiratory distress syndrome (ARDS) [4]. Diets rich in eicosapentaenoic acid (EPA, 20:5 n-3), found in fish oil, and γ-linolenic acid (GLA, 18:3 n-6), found in borage, evening primrose, and blackcurrant seed oils, appears to attenuate some of the pro-inflammatory effects of arachidonic acid [5-7].

Eicosapentaenoic acid is incorporated into and reduces the content of AA in cell membrane phospholipids. Eicosapentaenoic acid is available for enzymatic conversion to 3-series prostaglandins and 5-series leukotrienes [8]. These eicosanoids have anti-inflammatory properties and are considered less

biologically active than the 2-series prostaglandins and 4-series leukotrienes from AA [9]. γ-Linolenic acid is elongated to dihomo-γ-linolenic acid (DGLA), which can be converted to prostaglandin E_1 or 15-hydroxyeicosatetraenoic acid (15-HETE), which has been shown to attenuate leukotriene B_4 (LTB_4) formation by inhibiting 5-lipoxygenase [10]. Prostaglandin E_1 has also been shown to indirectly decrease leukotriene formation by the inhibition of 5-lipoxygenase [11]. The activation of neutrophils with lipopolysaccharide will prolong their survival *in vitro* [12]. This effect can be blocked with LTB_4 receptor antagonists, implying that LTB_4 plays a critical role in neutrophil viability. Leukotriene B_4 also is a potent recruiter of neutrophils to an inflamed site [13]. The ability to change the eicosanoid profile from pro-inflammatory AA metabolites to less biologically active EPA metabolites is beneficial in acute inflammatory conditions such as ARDS.

II. Rationale and Significance

Acute respiratory distress syndrome is an acute inflammatory process characterized by neutrophil accumulation, increased pulmonary capillary permeability, pulmonary edema, increased pulmonary vascular resistance, and progressive hypoxemia [14;15], all of which have been attributed to the release and metabolism of arachidonic acid to pro-inflammatory eicosanoids [16]. Diets containing n-3 and n-6 PUFA, such as EPA and GLA, have been shown to rapidly decrease the fatty acid concentration of arachidonic acid in cellular phospholipids, thereby reducing the potential amount of arachidonic acid available for metabolism [9;16;17]. Enteral nutrition with a diet containing EPA and GLA reduces pulmonary inflammation and improves gas exchange and clinical outcomes in patients with the ARDS [7]. The ability to attenuate inflammation by dietary intervention has been of great interest since a nutritional intervention is low risk to patients [16]. Our laboratory participated in a multi-center clinical study that showed that enteral nutrition with specialized diets containing EPA and GLA reduced the number of neutrophils in the bronchoalveolar lavage (BAL) fluid, reduced pulmonary inflammation, and improved clinical outcomes of patients with ARDS [16]. Our laboratory has also shown that diets containing EPA and GLA reduce the levels of leukotriene B_4 in the BAL fluid, as well as neutrophil accumulation in the lungs of endotoxic animals on experimental diets compared with endotoxic animals on control diets [9;18]. Thus, EPA and GLA have been shown to decrease lung microvascular

permeability and improve oxygenation, cardiopulmonary function, and to reduce pro-inflammatory eicosanoid synthesis in animal models of acute lung injury and in human ARDS [9;16]. These improvements may be a direct result of fewer neutrophils in the lung due to their reduced migration and/or increased clearance by alveolar macrophages.

I hypothesize that the anti-inflammatory effects of EPA and GLA may be due, in part, to induction of neutrophil apoptosis. I plan to use the human promyelocytic HL-60 cell line as a model for human neutrophils *in vitro*. HL-60 cells share many functional characteristics with peripheral blood neutrophils, such as containing azurophilic granules, and are, therefore, considered the preferred immortalized cell line model for studying human neutrophil responses *in vitro* [19;20]. Human neutrophils are short lived and will undergo apoptosis in 6-12 hours *in vitro*. HL-60 cells are an immortal cell line and do not spontaneously undergo apoptosis at an accelerated rate. Thus, HL-60 cells are a good substitute to measure the induction of apoptosis by fatty acids and are a good model for peripheral blood neutrophils [19]. The significance of this work is that elucidating if and how EPA and GLA are inducing apoptosis in HL-60 cells may lead to new strategies to increase neutrophil apoptosis that may provide an injury-limiting pathway in the resolution of inflammation such as that seen in ARDS.

III. Objectives

Objective 1: The primary goal of this dissertation is to determine the effect of eicosapentaenoic acid, γ-linolenic acid, and the combination of eicosapentaenoic and γ-linolenic acids on apoptosis in the human promyelocytic HL-60 cell line. The results obtained may help to explain the results observed in the ARDS study [16], as well as animal studies [9;18], in that there were fewer neutrophils and lower levels of LTB_4 in the bronchoalveolar lavage fluid of patients and animals with ARDS on the experimental diet than in those patients and animals on a control diet.

Objective 2: The second objective of this dissertation is to determine the effect of inhibitors of the lipoxygenase and cyclooxygenase enzymes on apoptosis in eicosapentaenoic acid, γ-linolenic acid, the combination of eicosapentaenoic and γ-linolenic acids, as well as arachidonic acid-treated HL-60 cells. The results obtained will determine if the eicosanoids products of these fatty acids are important in the regulation of apoptosis in HL-60 cells. Furthermore, the results will determine which branches of the eicosanoid metabolic pathways may be important in the regulation of apoptosis in HL-60 cells.

Objective 3: The objective of this dissertation is to determine the apoptotic gene profile of HL-60 cells treated with the combination of eicosapentaenoic acid and γ-linolenic acid. The results obtained will determine which apoptotic genes, both

pro- and anti-apoptotic are involved in EPA/GLA induced HL-60 cell apoptosis. Knowledge of these genes will allow the possible elucidation of the genetic mechanism via EPA and GLA induce apoptosis in HL-60 cells.

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Part 2

Literature Review

I. Acute Respiratory Distress Syndrome

Introduction to ARDS:

Acute respiratory distress syndrome (ARDS) was first described by Ashbaugh and colleagues in 1967 [1]. ARDS has several synonyms including, but not limited to: adult respiratory distress syndrome, shock lung, Da Nang lung, white lung syndrome, and wet lung syndrome [2]. Acute lung injury has been defined by the European-American Consensus Conference on ARDS to be a condition involving three key components. First, impaired oxygenation as defined as a ratio of partial pressure of arterial oxygen (PaO_2) to the fraction of inspired oxygen (FiO_2) that is $\leq$ 300 mm Hg regardless of how much positive end-expiratory pressure (PEEP) is used to provide respiratory support. Second, frontal chest radiograph detection of bilateral pulmonary infiltrates. Third, a pulmonary artery occlusion pressure of $\leq$ 18 mm Hg [3]. The definition of ARDS consists of the same three components except that the ratio of PaO_2 to FiO_2 must be less than 200 mm Hg regardless of PEEP [3;4]. When ARDS was first described in 1967 the mortality rate exceeded 95% [2]. The incidence of ARDS in the United States of America has been estimated at 150,000 cases per year and as recently as 1995 mortality still exceeded 50% [4;5]. Since 1967, ARDS has become recognized as a frequent cause of acute respiratory failure in both medical and surgical patients [6].

Etiology of ARDS:

Many disorders are associated with ARDS such as aspiration of gastrointestinal fluid or near drowning, drug toxicity, inhalation injury such as oxygen toxicity, smoke inhalation, hematologic disorders such as multiple transfusions, metabolic disorders such as pancreatitis, and neurological disorders such as brain tumor [2]. However, the most common disorders associated with ARDS are shock, trauma, and infection, particularly pneumonia and sepsis [2].

Pathology of ARDS:

The pathology of ARDS can be broken down into 3 specific phases; exudative phase, proliferative phase, and fibrotic phase [7-9]. The exudative phase consists of edema and hemorrhage. This phase typically occurs during the first week after the onset of respiratory failure [9]. Changes consisting of intra-alveolar hemorrhage, capillary congestion, and interstitial and alveolar edema can be detected via bright field microscopy [7;9-11]. Most of these changes occur through endothelial cell activation [12]. The distinct histologic features of this phase are the formation of eosinophilic hyaline membranes along the alveolar duct [9;10;13]. Injury to the hyaline membranes and, more importantly, the endothelial-epithelial barrier causes alveolar edema and increased cellular debris in the alveoli [9]. Much of this debris comes from necrosis of type I alveolar epithelial cells, which slough off, leaving only the basement membrane [9;14-16]. Endothelial cell injury with cellular swelling, increased pinocytotic vesicles, widening intercellular junctions, and disruption of the basement

membrane are visible with electron microscopy [17]. Typically, large numbers of neutrophils are present intracapillary. This is predominantly seen in diffuse alveolar damage secondary to sepsis and trauma [9;15;18]. Damage to the alveolar epithelial barrier allows interstitial fluid to flow freely into the alveolar spaces [9]. Increases in intra-alveolar edema and hemorrhage cause an increase in the diffusion distance. An increase in diffusion distance adversely affects the PaO_2/FiO_2 ratio. Typically the diffusion distance of gasses averages 2.2 μm in thickness [19].

The proliferative phase of ARDS consists of organization and repair [9]. By the third day after the clinical onset of ARDS, type II alveolar epithelial cells proliferate along alveolar septa in an attempt to cover the denuded basement membrane [9;17]. By the tenth day, fibrosis is apparent [7]. Type II alveolar epithelial cells manufacture surfactant and consist of 16% of the parenchymal cells of a normal lung [19]. These cells have a surface area of roughly 183 μm^2. In contrast, type I alveolar epithelial cells, which are flat to accommodate a proper diffusion distance of 2.2 μm, constitute only 8% of the parenchymal cells of a normal lung but have a surface area of roughly 5098 μm^2, or 28 times the surface area of type II cells [19]. The fibrosis of the lung occurs when fibroblasts and myofibroblasts proliferate and migrate through breaks in the alveolar basement membrane into the intra-alveolar exudate [9]. The fibroblasts convert the exudate into dense fibrous tissue by the deposition of collagen [9;20]. Therefore, the proliferation of type II cells and fibrosis of the lung transforms the

lung into a noncompliant end-stage organ and becomes a limiting factor in the survivability of ARDS [9].

The last phase of ARDS consists of end stage fibrosis and is known as the fibrotic phase [9]. This phase occurs three to four weeks after the onset of the clinical ARDS. It is characterized by widespread formation of collagenous tissue by fibroblasts causing thickened alveolar septa [2;9]. Intimal thickening and medial hypertrophy of the pulmonary arterioles also occurs during this phase [17;21]. This fibrosis reduces lung compliance, thereby decreasing the tidal volume and increasing the work of breathing, resulting in CO_2 retention [22].

Inflammation in ARDS:

The pathophysiology of ARDS is driven by an aggressive inflammatory reaction with the main players being neutrophils, macrophages, endothelial cells, and multiple mediator cascades [17;22]. Initiation of inflammation results in an increase in leukocyte production and rapid recruitment to the inflamed site [22]. Several of the mediator cascades that are activated during inflammation are the production of free radicals, chemokines, cytokines, acute phase proteins, complement, coagulation pathway components, and eicosanoids [23-25]. The most important of the cytokines involved in the process of inflammation are tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 [22]. TNF-α has been shown to play an important role in the coagulation response [26;27]. TNF-α levels are raised in the pulmonary microcirculation and bronchopulmonary secretions of patients with ARDS [28;29]. IL-8 is a proven chemoatractant for

neutrophils and IL-8 levels are raised in BAL fluid of patients who ultimately develop ARDS [22;30]. Excess production of inflammatory mediators, such as TNF-α and reactive oxygen species, by neutrophils has been observed in patients with ARDS [31-34]. The major leukocyte found in the bronchoalveolar lavage (BAL) fluid and histological specimens from patients with ARDS is the neutrophil [35].

Endothelium in ARDS:

The vascular endothelium is important in initiating and perpetuating the inflammatory response in ARDS [17]. Endothelial cell activation is a principal mechanism in the pathophysiology of ARDS [28]. Activation of the endothelial cells is defined as a change in phenotype or function that can be induced by various stimuli, including thrombin, cytokines, and bacterial products [27;28]. Endothelial cells express surface receptors for the thrombin, von Willebrand factor, intercellular adhesion molecule-1 (ICAM-1), and selectins following exposure to an activating stimulus [17;36]. Endothelial cells thrombin receptors bind thrombin, which regulates endothelial cell function [28]. Binding of thrombin causes endothelial cells to have a pro-thrombotic phenotype and increases endothelial cell permeability by producing gaps between adjacent endothelial cells, which leads to microvascular dysfunction and intravascular coagulation [12;17;28]. Von Willebrand factor is also released from stimulated endothelial cells [37]. Increased levels of von Willebrand factor antigen have been detected in patients with at-risk diagnoses for the development of ARDS [37]. ICAM-1 and

selectins are important components of the neutrophil-endothelial cell interaction [17]. These interactions lead to the sequestration of neutrophils within the pulmonary vasculature and subsequent tissue damage [9;17;28]. ICAM-1 interacts with the CD11/CD18 adhesion molecules expressed on the surface of neutrophils [37]. The expression of CD11/CD18 on neutrophils is up-regulated in the presence of inflammatory cytokines such as IL-1 and TNF-α [37]. Selectins are also up-regulated by inflammatory cytokines. E-selectin mediates endothelial cell adhesion to a variety of leukocytes and is endothelial cell-specific [37]. P-selectin is expressed in the Weibel Palade bodies of the endothelium and in the alpha granules of platelets [38]. P-selectin, expressed on the surface of endothelial cells, binds to the sialyl Lewis X chain on the surface of neutrophils [38]. Vasoactive substances, such as prostacyclin (PGI_2), endothelins, and nitric oxide, are also produced and released by the endothelium [17].

Macrophages in ARDS:

The macrophage plays a critical role in the development of inflammation in ARDS [17;39]. The alveolar macrophage is a resident cell within the lung and contributes greatly to the inflammatory response by releasing pro-inflammatory compounds such as eicosanoids, cytokines, and platelet activating factor (PAF) [17;39]. Macrophages release eicosanoids, implicated in the development of ARDS, derived from the cyclooxygenase and lipoxygenase pathways upon stimulation [39]. Macrophages are also responsible for removing apoptotic neutrophils by recognizing externalized phosphatidylserine on apoptotic cell

surfaces [40;41]. Alveolar macrophages also protect against local infection. Macrophages from patients with ARDS have defective phagocytosis and bacterial killing abilities [17]. These macrophage defects exacerbate the deleterious effects of ARDS by increased risk of infection and decreased clearance of neutrophils.

Neutrophils in ARDS:

Neutrophils account for less than 3% of total cells in BAL fluid from healthy patients. Conversely, 85% of the total cell population is neutrophils in BAL fluid from patients with ARDS [42]. Because of the neutrophils' destructive potential, they are thought to be the key player in the pathology of ARDS [43;44]. Neutrophils are attracted to an inflamed site by a variety of compounds, such as bacterial factors, complement components, lymphokines, peptides, immunoglobulin fragments, macrophage products, cell surface antigen-antibody reactions, interleukines, and lipids including prostaglandins and leukotrienes [33;45]. Neutrophils themselves are important sources of pro-inflammatory compounds [17]. In order for neutrophils to cause tissue damage, they must first migrate from the circulation to the target tissue area. Neutrophils change in both biochemical and adhesive properties upon stimulation to facilitate their movement [43]. Neutrophils diapedese and chemotax through a variety of interactions. Neutrophils roll along the endothelium due to interaction with selectins (e.g. L, P, E-selectin) and intracellular adhesions molecules (e.g. ICAM-1) [17;43;46]. Blockage of L-selectin with a monoclonal antibody prevented

neutrophil sequestration in alveolar capillaries in an endotoxemic rabbit model, demonstrating the importance of L-selectin in neutrophil migration [47]. Once the neutrophil has diapedesed, it is attracted to the inflamed site by a variety of pro-inflammatory compounds, such as leukotriene B_4 and IL-8 [30;48;49]. Neutrophils have a potent mechanism for phagocytosing and killing microbes [45]. Neutrophil pseudopodia engulf particles in a pouch of plasma membrane. This process is facilitated by opsonization of the phagocytosed particle [45]. Once the microbe or particle has been phagocytosed, it is subjected to digestion by potent enzymes and O_2 free radicals [45].

Oxygen metabolite-mediated injury is the likely insult provided by neutrophils in ARDS [50]. Both patients and animal with ARDS have increased levels of oxidative stress, as exhibited by increased blood lipid peroxide levels, increased exhalation of H_2O_2, and decreased blood and BAL glutathione levels [44;50]. Plasma protein thiol levels are significantly lower in ARDS patients who did not survive than in those who did, suggesting they suffer from severe oxidative stress [51]. Metnitz et al, 1999 [52] demonstrated that the anti-oxidants alpha-tocopherol, beta-caratene, selenium, and ascorbate were reduced in the blood of patients with ARDS compared with healthy volunteer blood. Additionally, plasma lipid peroxidation levels were significantly increased. The production of oxygen radicals ($^{\cdot}O_2^-$, H_2O_2, $^{\cdot}OH$) by neutrophils is referred to as oxygen or respiratory burst. This burst is associated with increased oxygen consumption, hexose monophosphate shunt activity, and chemiluminescence [45]. Many of the reactive oxygen species are most likely formed via the NADPH

oxidase complex [43]. Some of the most important mediators of neutrophil chemotaxis, activation, and survival are metabolites of arachidonic acid, the eicosanoids, particularly the leukotrienes and prostaglandins [49;53;54]. Thus, the eicosanoids are critical in the pathology of ARDS.

II. Eicosanoids and Essential Fatty Acids

Metabolism of Essential Fatty Acids:

Eicosanoids are 20 carbon fatty acid oxidized derivatives of essential fatty acids [55]. There are two families of essential fatty acids, the n-3 or ω-3 and the n-6 or ω-6. Certain fatty acids were found to be essential in 1929 by Burr and Burr [56]. They noted that rats eating a fat free diet developed a series of phenotypic changes including a scaly condition of the skin, swollen and scaly tail, and the tendency to lose hair from the face, back, and throat [56]. These fatty acids are considered to be essential because the human body cannot produce them and they must come from the diet [57]. The human body does not exhibit a desaturase enzyme that can alter the structure of the fatty acid beyond the ninth carbon from the carboxcylic end of the molecule. Thus, like any other essential nutrient, without it, disease will follow.

The parent compounds of the n-3 and n-6 fatty acids are α-linolenic acid (18:3 n-3) and linolenic acid (18:2 n-6), respectfully. These fatty acids can be elongated and desaturated via a series of enzymes (Fig. 2.2.1). The same enzymes metabolize both the n-3 and the n-6 fatty acids. The n-3 fatty acids can be converted into eicosapentaenoic acid (EPA, 20:5 n-3) and the n-6 fatty acids to arachidonic acid (AA, 20:4 n-6). It is important to note that fatty acids from the different n-families are not interconvertible [58]. Fatty acids that are of interest in this dissertation are EPA, AA, and γ-linolenic acid (GLA, 18:3 n-6). EPA is found in fish oils, AA exclusively in animal products, and GLA in borage, evening

Figure 2.2.1. The n-3 and n-6 families of fatty acids.

primrose, and blackcurrant seed oils [55;59;60]. Diets rich in AA lead to the formation of pro-inflammatory compounds while diets rich in EPA and GLA attenuate some of the pro-inflammatory effects of AA [55;61]. Pro-inflammatory products from AA most likely play an essential role in the development of septic shock, systemic inflammatory response syndrome, acute lung injury, and ARDS [62].

Polyunsaturated fatty acids, such as EPA and AA, are typically stored in the cell in the sn2 position of phospholipid membranes [63]. Newly ingested fatty acids enter into the phospholipid pools through the constant process of lipid remodeling occurring in the cell [63]. When a cell is stimulated, phospholipase A_2 (PLA_2) clips the fatty acid out of the sn2 possition yielding a free fatty acid and a lysophospholipid (Fig. 2.2.2.) [64]. This particular PLA_2 is a calcium-dependent phospholipase from the group IV family known as $cPLA_2$ [64-66]. $cPLA_2$ is specific for PUFA, particularly AA in the sn2 position of the phospholipids [66]. Once the PUFA from the sn2 position has been released by $cPLA_2$ it can be further metabolized by cyclooxygenase, lipoxygenase, or cytochrome P450 enzymes.

Cyclooxygenases:

The cyclooxygenase pathway consists of two primary enzymes, ubiquitously expressed prostaglandin H synthase-1 (PGH 1) or cyclooxygenase 1 (COX 1) and inducible prostaglandin H synthase-2 (PGH 2) or cyclooxygenase 2 (COX 2)

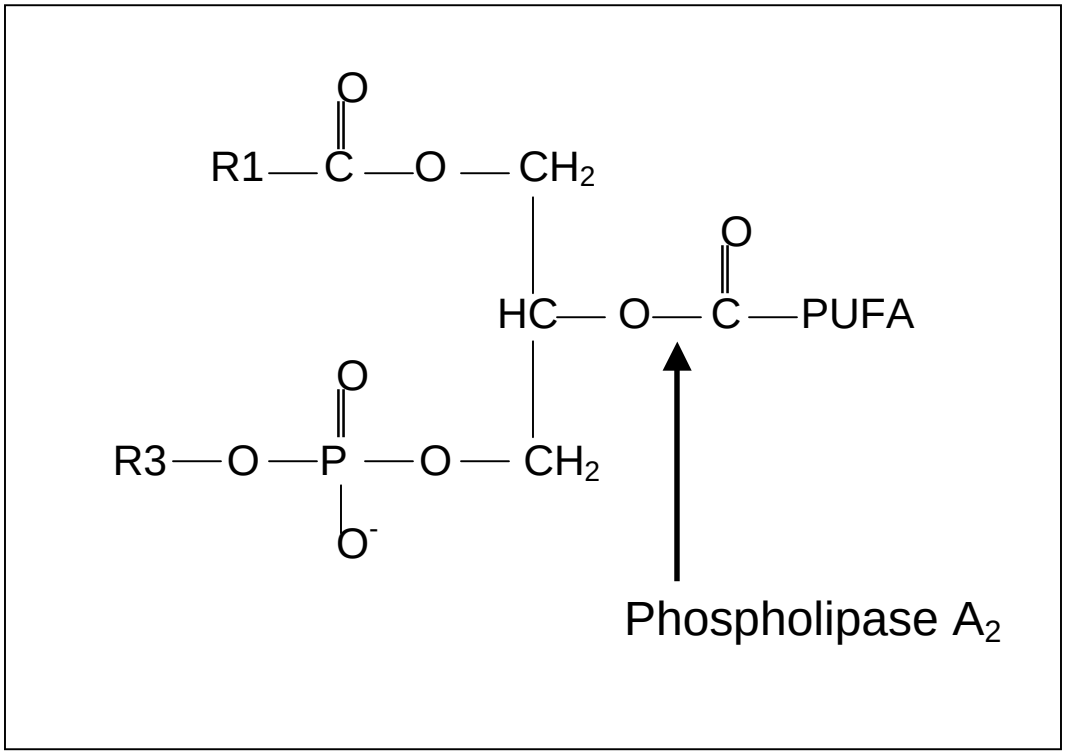

Figure 2.2.2. Schematic of the action of $cPLA_2$.[1]

[1]Adapted from Capper and Marshall, 2001 [66].

[67]. COX 1 is located on the luminal surface of the endoplasmic reticulum and COX 2 is located on the luminal surface of the nuclear envelope [68]. There are, however, conflicting reports as to where the isoforms are located. Several studies state that COX 1 is equally distributed between the endoplasmic reticulum and nuclear membrane, as well as that COX 1 and 2 are on both the inner and outer nuclear membrane in certain cell types [67;69;70].

COX 1 gene location is on chromosome 9 and COX 2 gene location is on chromosome 1. COX 1 gene is ~22 kb in length and does not contain a TATA box. COX 2 gene is ~8 kb in length and does contain a TATA box, while COX 2 promoter is highly inducible [68]. COX 1 contains 11 exons and 10 introns while COX 2 contains 10 exons and 9 introns [71]. COX 1 mRNA is 2.7 kb in size and COX 2 mRNA is 4.5kb in size [71]. COX 2 mRNA is much less stable than COX 1 mRNA. COX 1 mRNA is found to be constitutively expressed in most tissues in the body, while COX 2 mRNA is normally undetectable in most tissues [68]. The most obvious differences in COX 1 and COX 2 gene products is that COX 1 contains a 17 amino acid sequence on its amino end that COX 2 does not contain; and COX 2 contains an 18 amino acid sequence on its carboxyl end that COX 1 does not [72]. COX 1 gene product contains 576 amino acids while COX 2 gene product contains 587 amino acids [73]. The active site of COX 1 is a slightly different shape than the active site of COX 2 even though their crystalline structures are super-imposable.

Each form of COX carries out distinct reactions. COX 1 is, as stated before, constitutively expressed in many tissues. It is involved in cell-cell

signaling and homeostasis. It is active at relatively low levels when compared with COX 2. COX 2 is an inducible enzyme and is not normally present in unstimulated cells (exceptions: brain, testes, kidney) [74]. COX 1 expression is marginally affected by inflammatory stimuli while COX 2 expression is up-regulated by inflammatory stimuli. Since these two isoforms react under different cellular conditions, it is important that there are two forms. COX 1 has a Km ~20nm while COX 2 has a Km ~2nm for prostaglandin (PG) G_2 as a substrate. This means that COX 2 is activated with 1/10 the concentration needed to activate COX 1. Since COX 2 is primarily involved with mitogenesis and inflammation and is 10 times more responsive than COX 1, it would be harmful if constitutively expressed. Therefore, COX 2 becomes important in cells under some sort of duress (i.e., inflammation, mitogenesis).

The relative Km (μM) and Vmax (%) for COX 1 for arachidonic acid is 5.4μM and 105%, eicosatrienoic acid is 14μM and 74%, linoleic acid and is 12μM and 11%, and α-linolenic acid is 200μM and 7.6% [75]. The relative Km (μM) and Vmax (%) for COX 2 for arachidonic acid is 5.6μM and 106%, eicosatrienoic acid is 28μM and 128%, linoleic acid and is 27μM and 76.2%, and α-linolenic acid is 48μM and 74% [75]. γ-Linolenic acid and eicosapentaenoic acid are poor substrates for both COX 1 and 2 [74;75]. However, both acids are better substrates for COX 2 than for COX 1. Eicosapentaenoic acid is not converted to PGH_3 anywhere near as efficiently as arachidonic acid is converted to PGH_2.

COX 1 and 2 metabolize AA to PGG_2 and EPA to PGG_3 [67]. PGG_2 and PGG_3 are subsequently converted to PGH_2 and PGH_3, respectively, by the

peroxidase reaction of the COX enzyme [68;74]. PGH_2 can then be converted to a host of prostaglandins including PGI_2 (prostacylcin), PGE_2, and thromboxanes (TX) A_2 (Fig. 2.2.3) [74]. Metabolites of PGH_3 will be the same as PGH_2 except they have three double bonds instead of two and, subsequently, have the subscript 3 (i.e. PGE_3). PGH_2 can ultimately be converted to form many products including PGA_2, PGB_2, PGC_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2 (Fig 2.2.4). Prostaglandins derived from AA have been shown to be involved in the cardinal signs of inflammation: pain, swelling, and fever [76]. Eicosanoids derived from EPA counteract the responses of AA-derived eicosanoids [61]. TXA_2 is a potent platelet aggregator as well as a potent vasoconstrictor and bronchoconstictor produced in larger quantities by platelets than other tissues [74]. Conversely, TXA_3, derived from EPA, is a very weak platelet aggregator and vasoconstrictor [61;77]. PGI_2 is a potent vasodilator of vascular smooth muscle cells as well as a potent inhibitor of platelet aggregation [78]. PGI_3, derived from EPA, is just as biological active as AA-derived PGI_2 [77]. PGE_2 is one of the most pro-inflammatory prostaglandins. PGE_2 increases vasodilatation, increases vascular permeability, as well as modulates fever and pain [77]. PGE_3 is a weak vasodilator of vascular smooth muscle cells. Furthermore, dietary EPA reduces the levels of PGE_2 in human subjects [59]. Anti-inflammatory compounds can also be formed when GLA is metabolized by COX. GLA is rapidly elongated to dihomo-γ-linolenic acid (DGLA), which is metabolized by COX to form the monoenoic prostaglandins (PGE_1) [62]. PGE_1 is a potent vasodilator of pulmonary and systemic circulation, as well as inhibiting platelet aggregation,

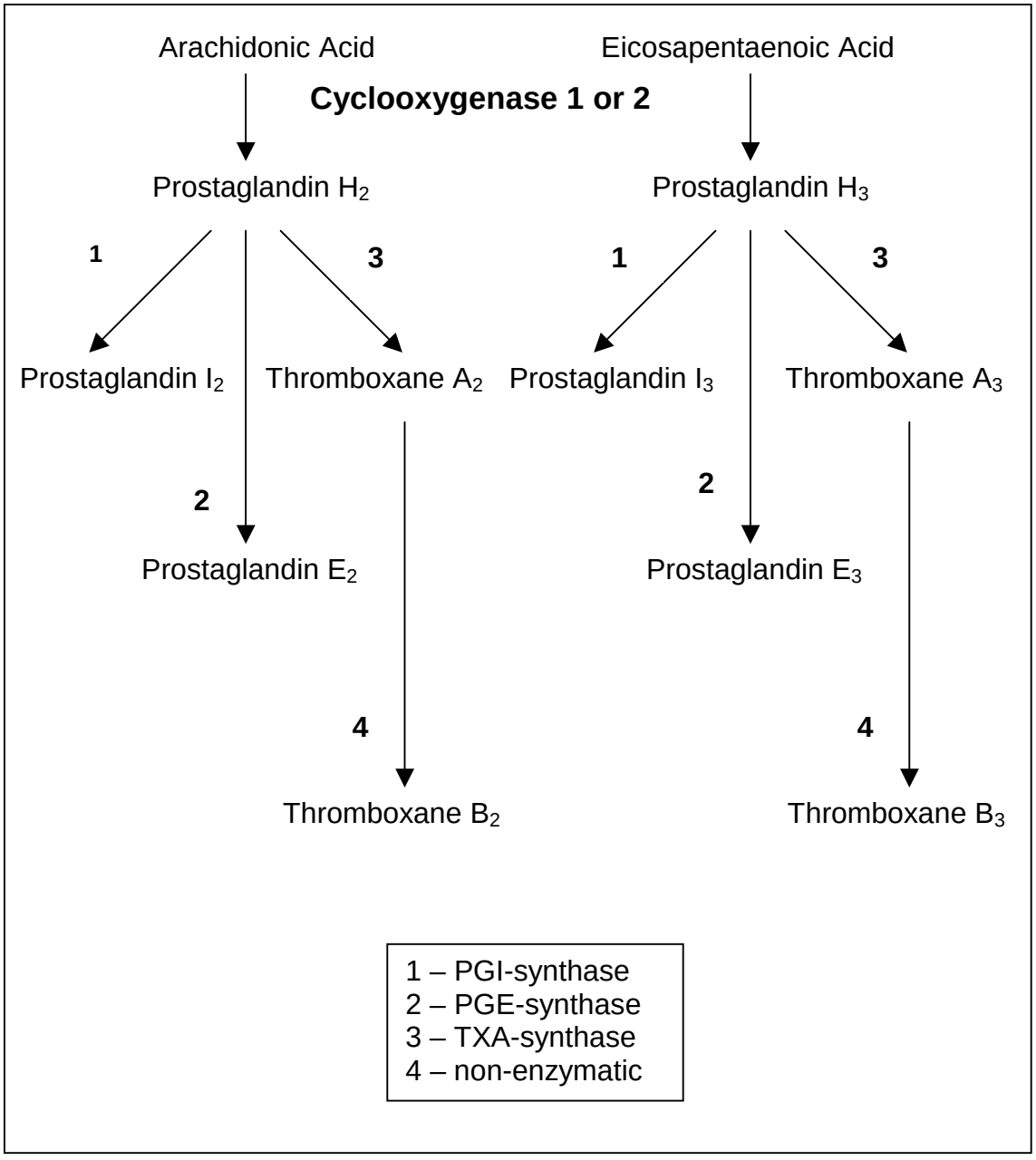

Figure 2.2.3. Schematic of cyclooxygenase pathway of eicosanoid formation.

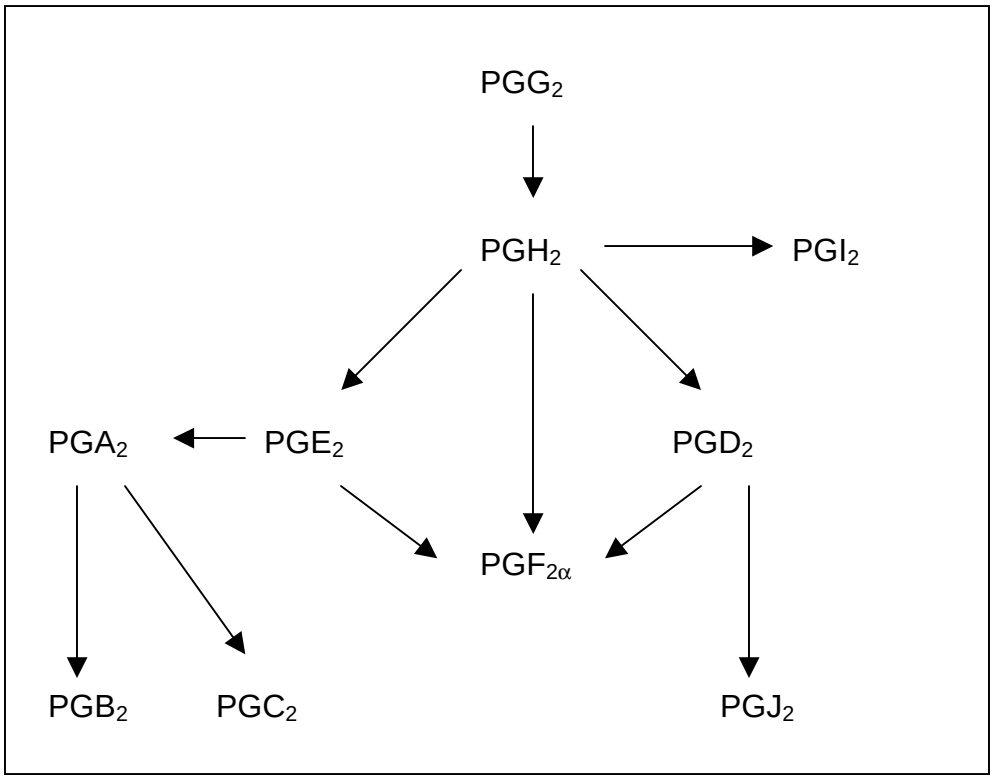

Figure 2.2.4. Schematic of prostaglandin products.[1]

[1]Adapted from Dr. Jay Whelan's Nutrition 602: Chemistry and Biology of Prostaglandins and Leukotrienes class notes.

neutrophil chemotaxis, oxygen free radical release, and phospholipase A_2 activity [57].

Recently, there has been the possible discovery of a third COX isoform, COX 3. It is thought that COX 3 does not produce pro-inflammatory compounds but produces anti-inflammatory compounds that are important in the resolution of inflammation [79]. Furthermore, the lack of this isoform may result in chronic inflammatory disorders such as rheumatoid arthritis [79]. COX 3 is made from COX 1 mRNA that retains intron 1 [80]. The intron that is left from the COX 1 mRNA introduces 30-34 amino acids into the COX 3 protein [80]. COX 3 mRNA is expressed in human cerebral cortex and heart as a 5.2 kb transcript [80]. COX 3 is selectively inhibited with various analgesic and anti-pyretic drugs such as acetaminophen, phenacetin, dipyrone, and antipyrine [80]. COX 3 is an isoform that is thought to explain why acetaminophen is an analgesic and anti-pyretic but not an anti-inflammatory compound [81;82].

Lipoxygenases:

The lipoxygenases are a group of dioxygenase enzymes that incorporate molecular oxygen into PUFA [83]. There are three groups of lipoxygenase (LO) enzymes in humans, 5-, 12-, and 15-lipoxygenase. Specifically there are 5 identified human lipoxygenases; human reticulocyte-type 15(S)-LO, human platelet-type 12(S)-LO, human 5(S)-LO, human epidermis-type 12(R)-LO, and human epidermis-type 15(S)-LO [84;85]. The number of the LO referes to the oxygenation site on a standard arachidonic acid molecule [83].

12-LO is a cytosolic protein that converts AA into12-hydroperoxyeicosatetraenoic acid (12-HPETE) [74]. 12-LO translocates from the cytosol to the membrane via a calcium-dependent mechanism as demonstrated in human erythroleukemia cells [86]. However, It has been demonstrated that 12-LO is inactive at the membrane [87]. 12-LO will also convert EPA to 12-hyrdoperoxyeicosapentaenoic acid (12-HPEPE). 12-HPETE is the starting material for the formation of the hepoxilins and trioxilins (Fig. 2.2.5) and 12-LO can also produce 15-HPETE under certain conditions [74;83]. 12-LO has also been shown to possess LTA synthase activity [83].

Human 12-LO consists of roughly 662 amino acid residues and has a molecular weight of roughly 75 kDa [83]. Human platelet-type 12-LO gene is located on chromosome 17 and the gene is 17kb with 14 exons. This gene contains a TATA-like box, as well as other transcriptional elements such as GC, AP-2, and CACCC [88]. If Vmax is set at 100% for arachidonic acid, EPA has a Vmax of 107%, 5-HETE 40%, 5-HPETE 3%, and 15-HPETE 2% [83]. Therefore, EPA is a better substrate for 12-LO than is AA.

The hepoxilins act in the release of intracellular calcium and the opening of potassium channels [83]. Hepoxilin A_3 has been thought to act as second messenger in blood platelets and neural cells [74]. Hepoxilin B_3 activity remains unknown [74]. The hepoxilins are short lived and rapidly hydrolyzed to form the trioxilins [74]. 12-LO has been shown to be present in the skin of rats, and when stimulated with bradykinin and platelet activating factor, both potent pro-inflammatory compounds, produced 12-HETE and hepoxilins [89]. Furthermore,

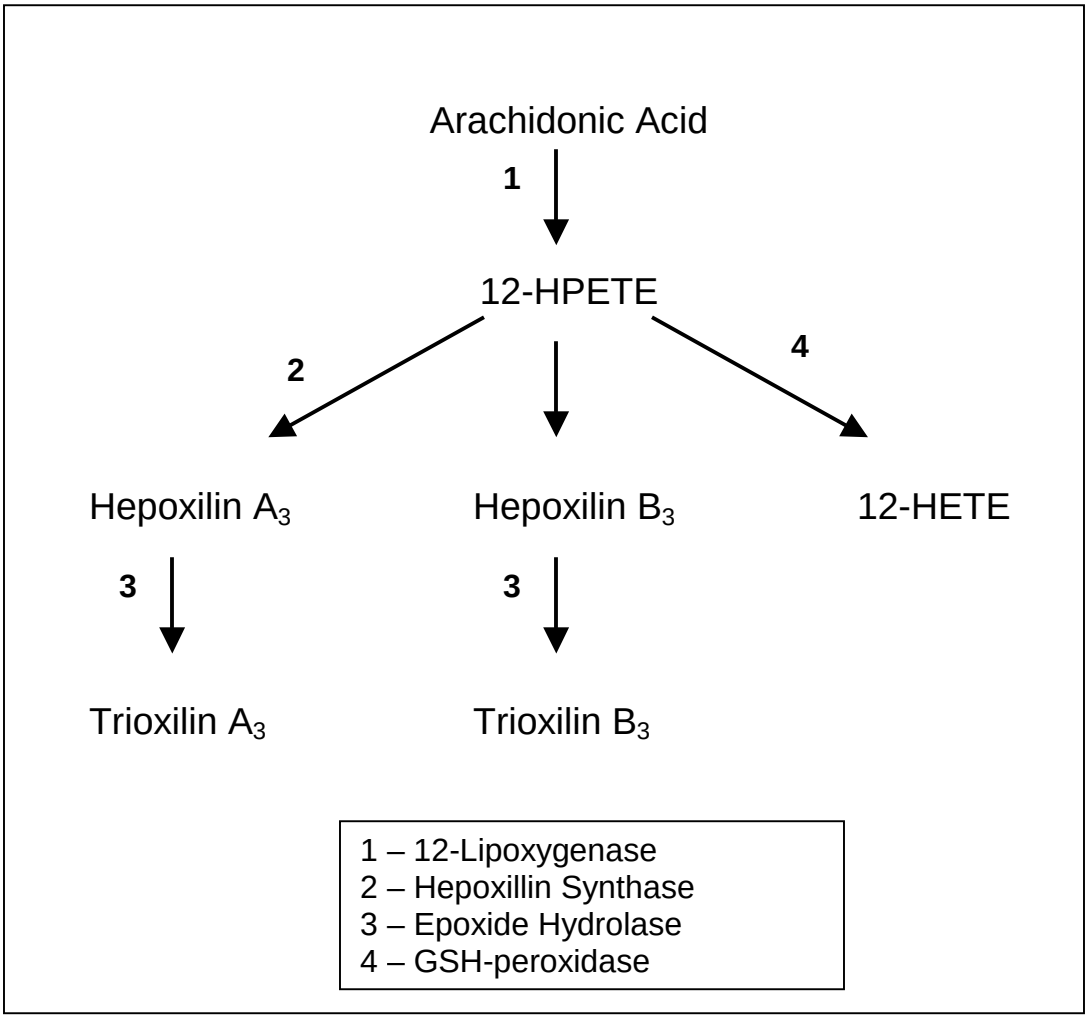

Figure 2.2.5. Schematic of 12-lipoxygenase metabolism.[1]
[1]Adapted from Yamamoto et al., 1997 [83].

12-HETE administered intradermally increased vascular permeability and acute inflammation [89]. Much work still needs to be done to elucidate the function of 12-LO products because physiological and pathological roles of 12-LO have not yet been established [83].

15-LO is a cytosolic protein that converts arachidonic acid in 15-HPETE [90]. There have been two different human 15-LO identified; 15-LOa (formerly called 12/15 LO or 15-LO1) and 15-LOb (formerly called 15-LO2) [91]. 15-LOa is highly expressed in bronchial epithelial cells and eosinophils [91]. 15-LOb is expressed in lung, skin, prostate, and hair roots [91]. There is evidence, such as seen with 12-LO, that 15-LO can translocate to the membrane in the presence of calcium [90]. 15-LO incorporates molecular oxygen at carbon 15 on arachidonic acid [91]. 15-LO is equally receptive to metabolize linoleic acid and incorporate molecular oxygen at carbon 13 [91]. 15-LO is not constitutively expressed in most human cells [90].

Human 15-LO consists of 661 amino acids and codes for a protein with a molecular weight of 75 kDa [90]. The 15-LO gene is located on chromosome 17 and has 14 exons and 13 introns [90]. This gene is very similar in size and placement to the human 12-LO gene, implying that they are closely related and recently diverged from each other on an evolutionary scale [90]. This gene has several promoter motifs including a TATTTA box, GC box, NF-1 binding site and a CCAAT element [90].

15-LOa metabolizes AA to form 15-HETE (Fig. 2.2.6) and LA to form 13-hydroxyoctadecadienoic acid (13-HODE) (Fig. 2.2.7) [91]. 15-LOb converts AA

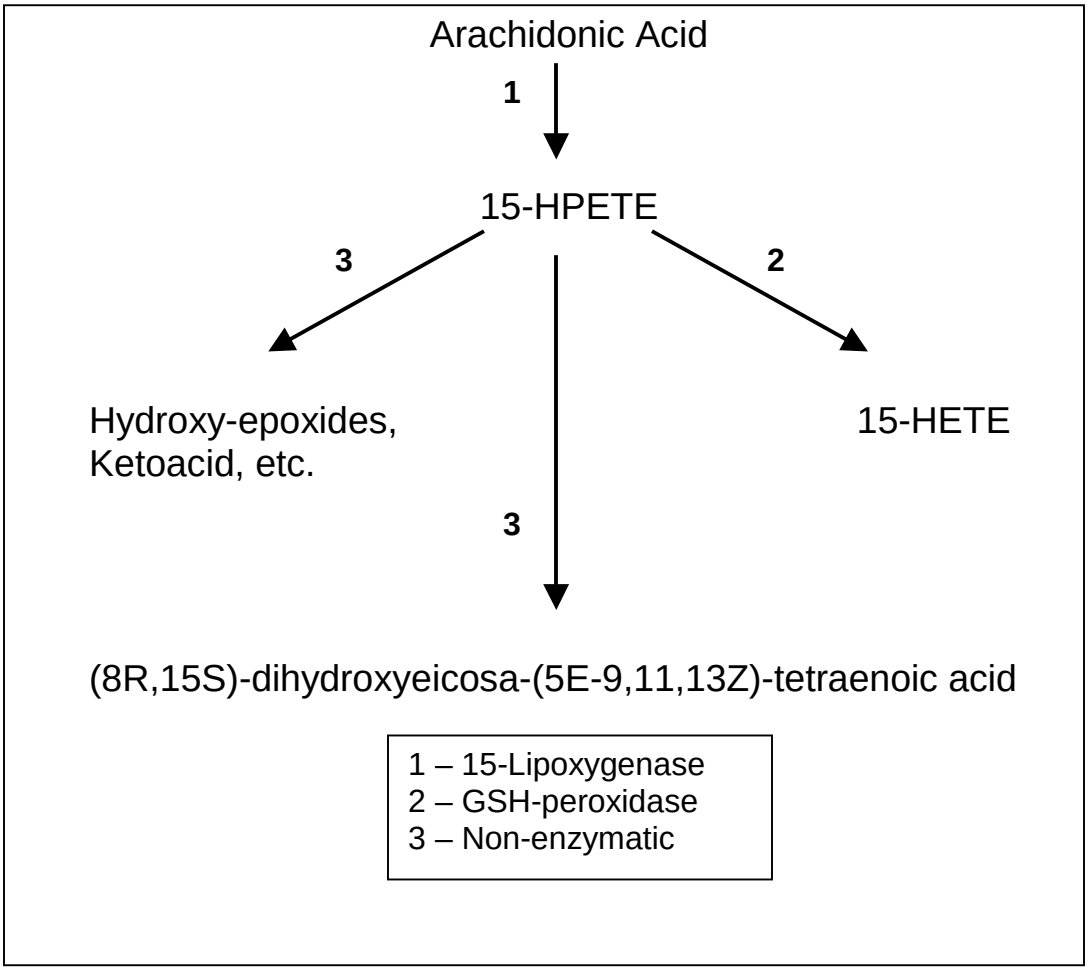

Figure 2.2.6. Schematic of 15-lipoxygenase metabolism.[1]

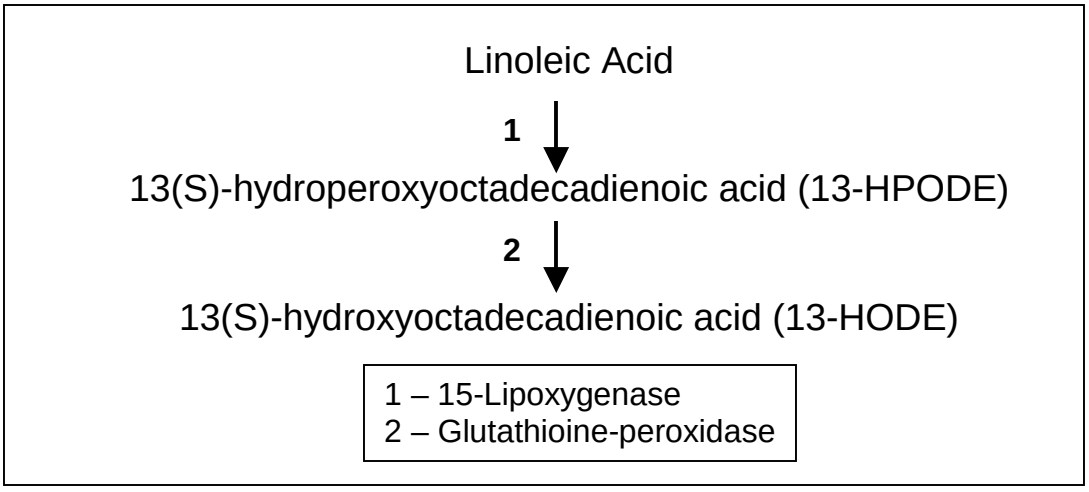

Figure 2.2.7. Metabolism of linoleic acid by 15-lipoxygenase.[1]
[1]Adapted from Piomelli, 1996 [74].

to 15-HETE but does not convert LA to 13-HODE effectively [91]. 13-HODE is involved in cellular signaling, such as when administered to fibroblasts, there is enhanced epidermal growth factor production [74]. AA metabolites of 15-LO are involved in inflammation and assist in the maturation of reticulocytes to erythrocytes [74]. 15-LO products have anti-inflammatory properties. 15-HETE inhibits leukotriene production by neutrophils as well as inhibits leukotriene B_4-induced chemotaxis of neutrophils and neutrophil migration across cytokine activated endothelium [91].

5-LO is cytosolic and is expressed in high levels in PMNs, monocytes, and macrophages [92]. Lower levels have been reported in tissues such as the brain [74]. When a cell is stimulated, cytosolic 5-LO translocates to the nuclear membrane [93]. There is recent evidence of a 5-LO that resides on the nuclear envelope [94]. This 5-LO also translocates to the nuclear membrane upon stimulation [93]. Once 5-LO has translocated to the nuclear membrane, it interacts with five lipoxygenase activating protein (FLAP). FLAP is an 18 kDa membrane-associated protein that is highly expressed in cells of myloid origin [92;95]. FLAP is critical in fatty acid metabolism by 5-LO [92]. FLAP acts as a transfer protein and delivers fatty acid released by $cPLA_2$ to 5-LO for metabolism [92].

The 5-LO amino acid sequence contains 672 or 673 amino acids and the monomers' molecular weight is between 72 and 80 kDa [96]. RNA from lung and leukocytes is 2.7 kb in size [96]. The 5-LO gene is located on chromosome 10 and contains 14 exons [97]. 5-LO activity is considered calcium-dependent.

However, evidence shows that there is some activity in the absence of calcium [96]. 5-LO is at full activation when calcium concentrations are between 4-10 μM [96].

5-LO metabolizes AA to form the 4-series leukotrienes and EPA to form the 5-series leukotrienes (Fig. 2.2.8) [98]. Eicosanoids from the 5-series leukotrienes are less biologically active than eicosanoids from the 4-series leukotrienes [39]. LTA_4 can go through LTA_4 hydrolase and form LTB_4. LTB_4 can be further modified by cytochrome P450 to produce 20-hydroxy-LTB_4 which again can be acted on by cytochrome P450 to form 20-carboxy-LTB_4 [74]. LTA_4 can also go through glutathione-S-transferase to produce LTC_4. LTC_4 can be further modified by γ-glutamyl transpeptidase to form LTD_4. Removal of the Gly form LTD_4 will create LTE_4. LTF_4 is created if the γ-Glu is placed back onto the LTE_4 [99]. Lipoxins are also derived from LTA_4. LTA_4 can be acted on by 12-LOX which will form 5(6)-epoxytetraene; this can be attacked by a hydroxyl at carbon 6 to produce lipoxin A_4 or attacked at carbon 14 to produce lipoxin B_4 [100]. 14,15-LTA_4 can also go through these same steps to produce both lipoxin A_4 and B_4 [101].

The cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, collectively were originally referred to as slow-reacting substance of anaphylaxis [102]. Cysteinyl leukotrienes have also been termed sulphido leukotrienes and peptidyl leukotienes [103]. Cysteinyl leukotrienes mediate smooth muscle contraction leading to vasoconstriction and bronchoconstriction [104]. They increase capillary permeability leading to plasma leakage as well as increase mucus

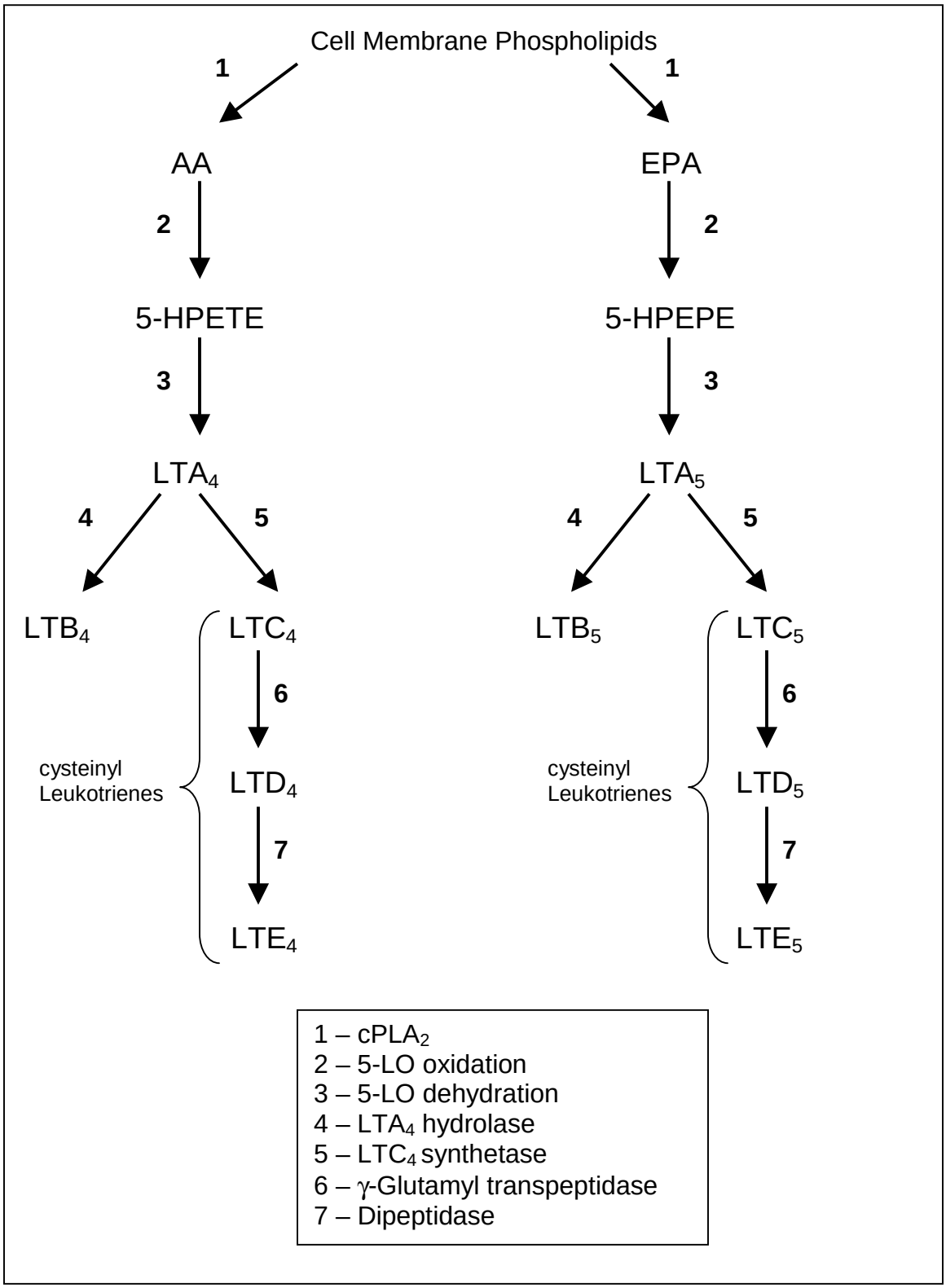

Figure 2.2.8. Schematic of the 5-lipoxygenase pathway.[1]

[1]Adapted from Brooks and Summers, 1996 [98].

secretions [76;103]. The cysteinyl leukotrienes have also been implicated in decreasing blood pressure by reducing myocardial contractility and coronary blood flow, as well as causing the release of luteinizing hormone from rat anterior pituitary cells [104].

LTB_4 has been shown to be important in promoting neutrophil chemotaxis and adhesion to vascular endothelium [76]. LTB_4 has also been shown to increase enzyme release, degranulation, and generation of superoxide in neutrophils [103;104]. LTB_4 mobilizes calcium pools and increases uptake of extracellular calcium, increases intracellular cAMP levels, and increases vascular permeability. LTB_4 plays a critical role in neutrophil viability and chemotaxis [49;54]. Recently, LTB_4 has been shown to be very important in promoting cell survival *in vitro*. Neutrophil survival was increased in the presence LPS. This survival was reversed when LTB_4 inhibitors were co-incubated with the neutrophils [105]. Leukotrienes derived from EPA are far less pro-inflammatory than are leukotrienes derived from AA. Furthermore, EPA derived leukotrienes compete for binding sites with AA-derived leukotrienes [106]. It is apparent that LTB_4 is intimately involved in inflammation, while the cysteinyl leukotrienes are intimately involved with allergy and asthma.

The formation of lipoxins occurs through the metabolism of multiple lipoxygenases [74]. The lipoxins can be formed from three basic starting materials: LTA_4, 15(S)-HPETE, and 15(R)-HPETE as shown in figure 2.2.9. The lipoxins are important in a variety of areas. The lipoxins will decease adhesion and transmigration of neutrophils and act as counter-regulatory signals in the

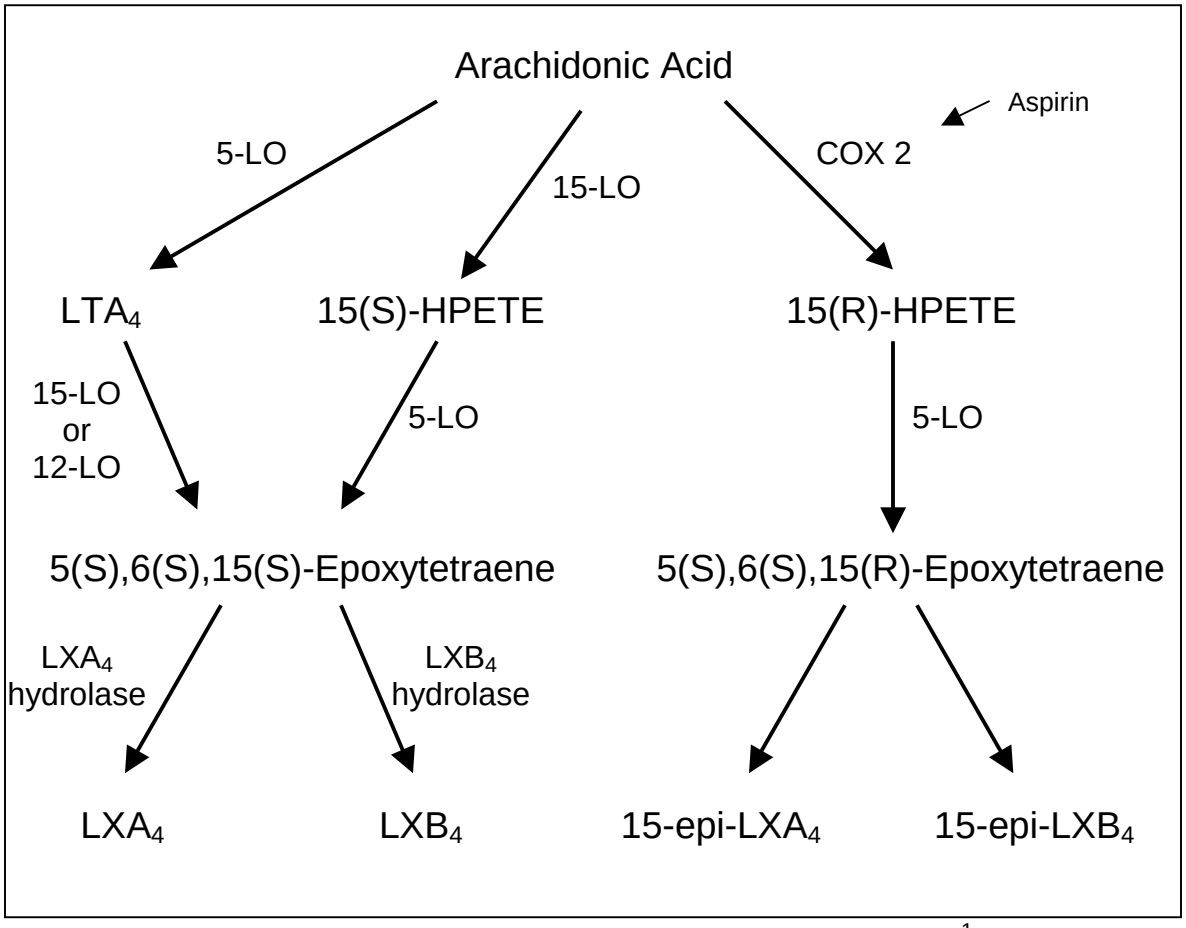

Figure 2.2.9. Schematic representation of the formation of lipoxins.[1]

[1]Adapeted form Serhan, 1997 [100], Serhan et al., 2000 [108].

resolution of inflammation [107]. They inhibit neutrophil chemotaxis induced by LTB_4 and fLMP [100]. They inhibit the endothelial cell leukocyte interaction, thereby reducing adhesion and transmigration [91]. Lipoxin A_4 stimulates the phagocytosis of apoptotic neutrophils by macrophages to aid in the resolution of inflammation [109]. Lipoxin A_4 has been shown to have spasmogenic activities. It will cause contraction in guinea pig lung strips. However, it does not cause contraction in the ileum of the guinea pig [104]. Lipoxin A_4 can act at the LTC_4-LTD_4 receptor as an agonist and inhibit LTD_4, and induce inositol-1,4,5-triphosphate (IP_3) generation. Lipoxin A_4 has been shown to activate protein kinase C (PKC) and act as an intracellular signal [101]. The lipoxins may act as immunoreglators, as well as be involved in wound healing; but, most importantly, they are involved in the resolution of inflammation [107]. Lipoxin A_5 and B_5, derived form EPA, are as biologically potent as AA-derived lipoxins A_4 and B_4 [110].

Aspirin already has a great anti-inflammatory ability just through its inhibition of the COX enzymes. Charles Serhan has described a unique attribute that aspirin has to further combat inflammation called aspirin-triggered lipoxins [100;108]. When aspirin inhibits COX 1, it cannot make any products. However, when aspirin inhibits COX 2, it will preferentially convert arachidonic acid to 15(R)-HETE instead of PGG_2. 15(R)-HETE is then converted to 15-epi-lipoxins (LX) via 5-LO (Fig. 2.2.9) [100]. 15-epi-LXA_4 has been shown to inhibit the recruitment of neutrophils to inflamed tissues. 15-epi-LXA_4 has most of the same biological activities as the other lipoxins previously discussed. Neutrophils are

known to increase tissue damage and exacerbate the inflammatory response. Neutrophils will create vascular injury; which, in turn, will lead to increased vascular permeability, followed by edema, and recruitment of more neutrophils. These neutrophils will, in turn, release more LTB_4 and the vicious cycle will continue. Serhan has demonstrated that inflammatory stimuli (e.g. LPS, fLMP) will induce COX 2 to produce 15(R)-HETE when in the presence of aspirin [100]. The 15-(R)-HETE will be modified by neutrophils as described above to form the aspirin-triggered lipoxins. The aspirin-triggered lipoxins will inhibit the movement of the neutrophils, thereby limiting the tissue damage and resolving inflammation [100;108].

Cytochrome P450:

Cytochrome P450 is a heme protein that participates in monooxgenase reactions [111]. It is found in the smooth endoplasmic reticulum and mitochondria, although most cytochromes are located in the mitochondria. It can react with O_2 and carbon monoxide [112]. One can distinguish cytochrome P450 from other cytochromes in that the carbon monoxide complex of the reduced form of cytochrome P450 absorbs light at 450nm [112]. Hence, it was given the name cytochrome P450.

Cytochrome P450 functions in four major areas: metabolism of fatty acids and their derivatives, production of steroid hormones, inactivation or activation of therapeutic drugs (“xenobiotic metabolism”), and conversion of chemicals to highly reactive molecules [113]. There are a variety of different isoforms of

cytochrome P450s and their absorbance spectra range from 446-452nm. The general reaction that is involved with cytochrome P450 involves NADPH and molecular oxygen ($NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH$) [113]. Cytochrome P450 is the terminal electron acceptor in the electron transport chain. Cytochrome P450 is involved in the formation of the epoxyeicosatrienoc acids (EETs), the hydroxyeicosatetraenoic acids (HETEs), and ω oxidation products from arachidonic acid [111]. During the formation of the EETs, an epoxide ring is formed. This ring is unstable and breaks down to alcohols and forms the dihydroxyeicosatrienoic acids (DHTs) [74]. All of the cytochrome P450 isoforms are capable of forming EETs. They all have regio-specificity and preferred stereo-specificity [111]. The isoforms locations in the body are tissue-specific. For example, CYP2C is highly expressed in the liver and kidney while CYP2J is highly expressed in the heart [113]. Many factors influence the expression of the isoforms. Pentabarbital increases CYP2B expression, fasting decreases CYP2C11 expression, and dietary salt increases CYP2C23 expression [111;113].

EETs are important mediators of endothelium-dependant relaxation in coronary microvessels [111]. EETs have also been implicated in activation of calcium-dependent potassium channels in vascular smooth muscle, as well affecting calcium channel activity in cardiac muscle cells [111;114]. 20-HETE has the opposite effect and inhibits calcium-activated potassium channels [111]. EETs are mediators of vascular tone [115]. 11,12-EET has important anti-inflammatory properties in that it prevents leukocyte adhesion to the vascular wall

[116]. More needs to be learned about cytochrome P450 products and their potential to possess anti-inflammatory properties.

Cell-Cell Interaction in Eicosanoid Formation:

Cell-cell and cell-tissue interactions are important in producing eicosanoids. This interaction is lost many times in *in vitro* homogeneous cell cultures. Cell-cell interactions are involved in the making of lipoxins. It was initially believed that more than one cell type may be involved in the production of the lipoxins since it required two lipoxygenation steps for their synthesis. One example is the pathway for biosynthesis between the neutrophil and platelet. The neutrophil houses the 5-LO and the platelet houses the 12-LO, both of which are necessary to produce lipoxins [108]. Greater than 50% of the LTA_4 formed by the neutrophil is released into the extracellular compartment. Neutrophil-adherent platelets pick up the LTA_4 and convert it, via 12-LOX, to the lipoxins [108]. It is important to note that the platelet and the neutrophil must be adherent for lipoxin synthesis to be complete [108]. Another case of lipoxin synthesis that requires tissue-cell interaction is in the synthesis of the aspirin-triggered lipoxins. When COX 2 is activated in the presence of aspirin in the endothelium or epithelium by pro-inflammatory cytokines, it will produce 15(R)-HETE. 15(R)-HETE is released and then converted to 15-epi-lipoxins by adherent leukocytes [100]. Cells must be adherent to the initial tissue or cell for complete conversion [100]. Cell-cell interaction between the PMNs and platelets in the formation of lipoxins is also important [101].

Another example of cell-cell interactions in the formation of eicosanoids is when there is co-incubation of human epidermis and neutrophils. When these two cell types are incubated together, there is a 90% increase in the cysteinyl leukotrienes when compared to incubation of neutrophils alone. This indicates that the epidermis is transforming the LTA_4 from neutrophils into cysteinyl leukotrienes [103]. This is not to say that cysteinyl leukotrienes would not be formed in a homogeneous culture of neutrophils, but their production would be dramatically less.

Benefits of EPA and GLA:

There have been many studies demonstrating the anti-inflammatory properties of dietary EPA and GLA [55;117-119]. As discussed previously, GLA is rapidly elongated to dihomo-γ-linolenic acid (DGLA), which can be converted to a variety of anti-inflammatory compounds. DGLA, alone, suppresses leukotriene and prostaglandin biosynthesis through competition with AA for binding sites on LO and COX [62]. DGLA is converted to PGE_1 via the COX enzymes [57]. PGE_1 inhibits platelet aggregation, dilates blood vessels and reduces blood pressure, and stimulates cyclic AMP formation that inhibits $cPLA_2$ release of pro-inflammatory AA [57]. PGE_1 also inhibits neutrophil chemotaxis and oxygen free radical release [57]. DGLA can be converted to 15-OH-DGLA, which is an inhibitor of the 5- and 12-LO enzymes [57]. This reduces the production of pro-inflammatory compounds such as LTB_4. Additionally, neutrophils do not contain Δ-5 desaturase, the enzyme that converts DGLA to AA (Fig. 2.2.1), and,

therefore, accumulate DGLA rather than AA in cellular phospholipids [120]. Thus, when a neutrophil is stimulated, $cPLA_2$ will release more anti-inflammatory DGLA than normal.

Dietary EPA has tremendous anti-inflammatory properties. As previously discussed, eicosanoids derived from EPA are less biologically active than eicosanoids derived from AA [39]. There are a few key exceptions: lipoxins derived from EPA are just as potent as lipoxins derived from AA [110]. Furthermore, lipoxins are considered to anti-inflammatory [107]. PGI_3 derived from EPA, is as biologically active as PGI_2, derived from AA [77]. PGI_2 is thought of as an anti-inflammatory prostaglandin [78]. Furthermore, EPA inhibits Δ-6 desaturase, a key enzyme in the production of AA from linoleic acid. EPA competes with AA for incorporation into membrane phospholipids; increasing EPA in the diet reduces the AA content of phospholipids membranes [60]. EPA will compete with AA for binding sites on COX and LO. Additionally, EPA is a better substrate for 12-LO [83]. It is clear that dietary EPA and GLA are important in the regulation of inflammation and that these fatty acids are capable of reducing inflammation in both acute and chronic situations. Furthermore, both EPA and GLA have been shown to induce apoptosis in certain cell types and the ability to induce apoptosis in neutrophils may lead to a rapid resolution of inflammation [121-123].

III. Apoptosis

Apoptosis:

Apoptosis plays a critical regulatory role in the control of the size of cell populations, normal tissue homeostasis, and resolution of inflammation [124;125]. Apoptosis is a closely controlled cell process. Out of control apoptosis can lead to diseases, such as neurodegenerative diseases, and lack of appropriate apoptosis can contribute to cancer [126]. Cellular control of apoptosis is very complex and our understanding of this process continues to grow every day. Yet there is still a lot to be learned on the topic. Cellular apoptosis can be broken down into two distinct pathways: death receptor pathway or the mitochondrial pathway [127]. The executioners of apoptosis in both the death receptor pathway and the mitochondrial pathway are the cysteine aspartate-specific proteases (caspases) [127]. All caspases have an active site, containing a cysteine, that cleaves substrates after an aspartic acid residue (Asp-Xxx) [128]. Caspases are initially synthesized as inert zymogens [128]. The activation of caspases works through a caspase cascade starting with the initiator caspases.

Death Receptor Pathway of Apoptosis:

In the death receptor pathway, the receptors are from the tumor necrosis factor (TNF) family (Fig 2.3.1). These receptors include TNF receptor 1, DR3/WSL, DR6, TRAIL/Apo-2L, and Fas/Apo-1 (CD95) [129]. Once a ligand binds, for

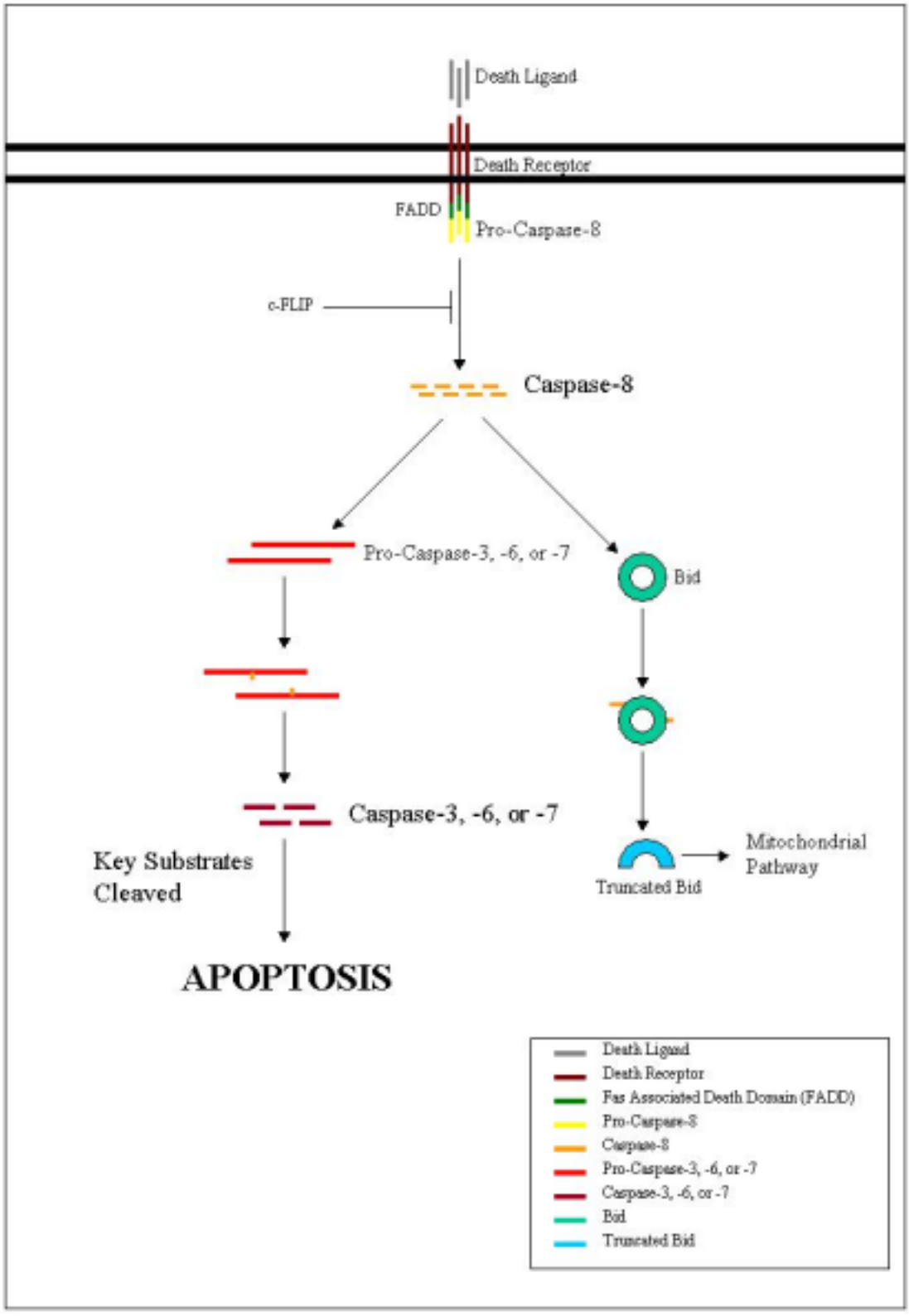

Figure 2.3.1. Schematic of the death receptor pathway of apoptosis.

example, Fas ligand to the Fas receptor, a series of cascading events occurs. First, this interaction causes the formations of an intracellular death-inducing signaling complex (DISC). This complex forms on the death domain [127]. The Fas-associated death domain recruits pro-caspase-8, the first of the death receptor pathway caspase cascade. Pro-caspase-8 is activated through cleavage via other pro-caspase-8s. This occurs when 2 or more pro-caspase-8s have aggregated at the Fas-associated death domain through a process known as induced proximity [127;128]. Caspase-8 is considered an initiator caspase. Caspase-8 activation can be blocked by the degenerate caspase homologue c-FLIP [128]. Caspase-8 activates the executioner caspases, caspase-3, -6, and -7 through cleavage of the pro-caspase forms [127]. The most prevalent caspase in the cell is caspase-3 [127]. Procaspase-3 (inactive caspase) exists as a 32 kDa protein. Once pro-caspase-3 is cleaved, it becomes active and yields a heterodimer consisting of p11 and p20 subunits [130]. Interestingly, this cleavage occurs at an Asp-Xxx site, inferring that caspases have autocatalytic activation [128]. Caspase-3 is considered the workhorse of apoptosis. Activated caspase-3 will cleave many proteins, such as cytoskeletal proteins, and proteins involved in DNA repair, such as polyADP-ribose polymerase (PARP) [130]. Caspase-3, along with caspases-6 and -7, are referred to as the “executioner caspases” [127].

Mitochondrial Pathway of Apoptosis:

The mitochondrial pathway of apoptosis is different in initiation than from the death receptor pathway (Fig 2.3.2). The mitochondrial pathway is usually initiated via some sort of cellular stress. For example, DNA damage may lead to apoptosis through the mitochondrial pathway. At this point in time, there are thought to be more players in mitochondrial apoptosis than there are in the Fas-Fas ligand death receptor pathway. Proteins from the Bcl-2 family primarily control the mitochondrial pathway. Bcl-2 family members can be broken down into two groups, pro-apoptotic and anti-apoptotic. A few of the pro-apoptotic members are; Bax, Bak, Bok, and Bid and a few of the anti-apoptotic members are; Bcl-2, Bcl-XL, Bcl-W, and Mc1-1 [127;128]. Bcl-2 and Bcl-XL are on the outer mitochondrial membranes while the pro-apoptotic proteins are cytosolic or can be present on the membrane [127]. Translocation of the pro-apoptotic Bcl-2 family members to the mitochondrial membrane initiates the apoptotic response. The pro- and anti-apoptotic Bcl-2 proteins are in a constant tug of war to determine if the cell is going to live or die. Once enough pro-apoptotic Bcl-2 members have congregated on the mitochondrial membrane, the cell reaches the point of no return. Most of the pro-apoptotic members in the cytosol are in the inactive form. They are activated through a variety of mechanisms, including proteolysis and dephosphorylation [128]. Once enough pro-apoptotic Bcl-2 family members have congregated to over-power the anti-apoptotic Bcl-2 family members, the mitochondria releases all of its cytochrome c. Cytochrome c is quickly released from all mitochondria in the cell [127]. Pro-apoptotic Bcl-2s are

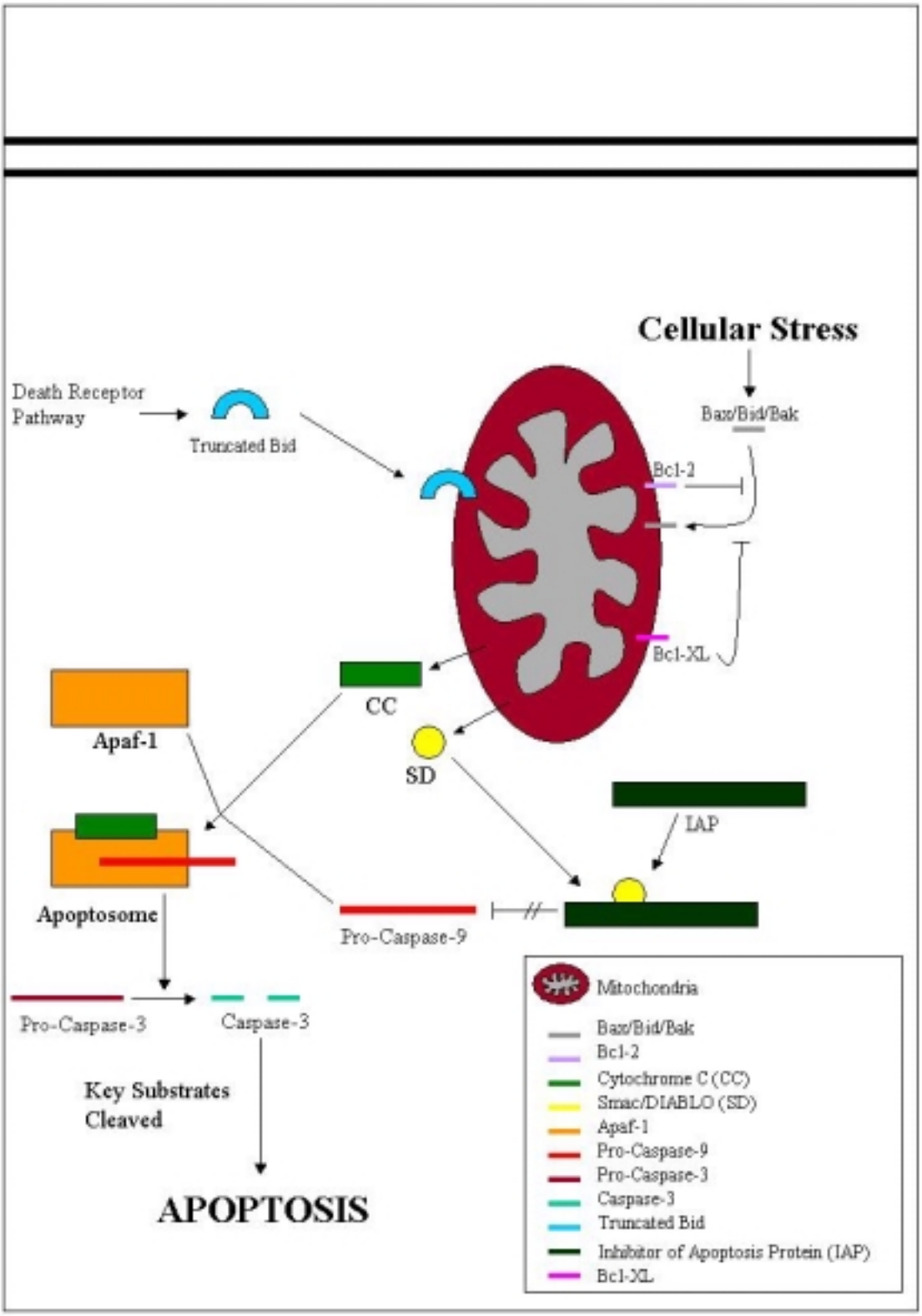

Figure 2.3.2. Schematic of the mitochondrial pathway of apoptosis.

thought to form large pores in the mitochondria to facilitate the release of the cytochrome c [128]. Pro-apoptotic Bcl-2 has a structural similarity to the pore-forming subunits of dipthera toxin [128]. Other proteins, such as second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO), are also released from the mitochondria at the same time as cytochrome c [127]. Smac/DIABLO further encourages the completion of apoptosis by inhibiting inhibitors-of-apoptosis (IAP) proteins [128]. Cytochrome c combines with apoptotic protease-activating factor-1 (Apaf-1) and pro-caspase-9 to form apoptosomes [127;128]. Unlike other caspases, pro-caspase-9 cannot be activated by simple cleavage, rather it must be part of the apoptosome [127]. Once caspase-9 is active. it can then cleave pro-caspase-3 and rest of the pathway is the same as the death receptor pathway. The only other connection between the two pathways is that caspase-8 can cleave Bid and truncated Bid translocates to the mitochondria and causes the release of cytochrome c [128]. The majority of the time the two pathways of apoptosis are distinct in the initiation and only one pathway will proceed [128].

Morphology of Apoptosis:

There are several distinct morphological characteristics of cellular apoptosis. Kerr et al. [131] first described morphological features via electron microscopy in 1972. Cells shrink and the cytoplasm condenses and the cell will detach from the surrounding tissue [132]. Microvilli appear and cell density increases as cytoplasmic organelles compact [133]. The cell continues to degrade through a

process known as budding when small parts of the cell break off and are contained within a cellular membrane to form apoptotic bodies [134]. The chromatin in the nucleus condenses and eventually begins to separate [132]. Condensed pieces of chromatin are often observed within apoptotic bodies [132]. Surrounding tissues and phagocytic cells, such as macrophages and dendritic cells, typically phagocytose apoptotic bodies [132]. If an apoptotic cell is not phagocytosed it will continue to degrade via secondary necrosis [134].

Some of the best described biological markers of apoptosis are internucleosomal DNA fragmentation, caspase activation, cytochrome c release, and externalization of phosphatidylserine (PS) [126;132]. Internucleosomal DNA fragmentation is considered a hallmark of late apoptosis. Cellular DNA is degraded in the internucleosomal regions into double-stranded DNA fragments of 180-200 base pairs by endogenous DNAase, giving a characteristic banding upon gel electrophoresis [132;134]. Activation of caspases and release of cytochrome c are both important aspects of the pathways of apoptosis, as previously discussed. In early apoptosis, PS, which is found on the inner leaflet of the cell membrane, translocates to the outer leaflet of the cell membrane while the cell maintains full membrane integrity [135]. *In vivo*, externalized PS is a physiological marker that macrophages use to recognize and engulf apoptotic cells before they degrade via secondary necrosis [41].

Apoptosis vs. Necrosis:

Degradation via apoptosis is a controlled and ordered process. Apoptosis typically affects single cells that are scattered throughout a tissue. Conversely, necrosis affects groups of contiguous cells [133]. Apoptotic cells are unique in that as they degrade they do not spill their cytoplasmic organelles into the extracellular environment. Rather, apoptotic bodies are formed that are rapidly phagocytosed. This rapid clearance of apoptotic bodies allows cell death via apoptosis to occur without inducing an inflammatory response [136]. In apoptosis, cells first shrink; in necrosis, cells first swell, causing the plasma membrane to break and the cell lyses [137]. Necrotic cells degrade rapidly, losing membrane integrity via lysis. Loss of membrane integrity allows the cell to spill all its contents, including proteolytic enzymes that cause local tissue damage and induce an inflammatory response [138]. Necrosis was originally called oncosis, which is derived from *oncos*, meaning swelling [137]. Most of the biochemical markers of apoptosis previously described are absent in a necrotic cell. It is clear that apoptosis and necrosis are two distinct forms of cellular death. Apoptosis is considered a normal non-harmful event while necrosis is indicative of a pathological process [137].

Apoptosis and Polyunsaturated Fatty Acids:

Polyunsaturated fatty acids have been shown to induce apoptosis in a variety of cell types *in vitro*. Apoptotic cell death is induced in the pancreatic caner cell line Mia-Pa-Ca-2 with treatment of a variety of n-3 and n-6 PUFA. Oleic (n-9),

linoleic (n-6), α-linolenic (n-3), and γ-linolenic (n-6) acids only slightly induced apoptosis in Mia-Pa-Ca-2 cells [123]. However, the longer chain PUFA, arachidonic (n-6), *cis*-parinaric (n-3), eicosapentaenoic (n-3), and docosahexaenoic (n-3) acids all induced significant amounts of apoptosis [123;139]. The relative cytoxic potencies of various PUFA are related to the carbon chain length, the longer the chain the more potent the induction of apoptosis [123;140]. EPA has also been shown to induce apoptosis in Walker 256 rat carcinoma cells [141]. γ-Linolenic acid has been shown to induce apoptosis in the human lymphoblast cell lines TK6 and WTK1, chronic myeloid leukemia (CML), human cervical cancer cell line HeLa, Walker 256 rat carcinoma cells, and glioma spheroid cell lines U87, U373, and C6 [141-145]. Arachidonic acid (AA) has been shown to induce apoptosis in the human lymphoblast cell lines TK6 and WTK1, and chronic myeloid leukemia (CML) [142;143]. Arachidonic acid induced apoptosis has also been demonstrated in human neutrophils [146]. Lastly, PUFA of the n-3 (EPA) and n-6 (AA) families have been shown to induce apoptosis in human promyelocytic HL-60 cells [147-149].

The proposed mechanism by which PUFA induce apoptosis in these cell types is debated and differs for each cell line. Several studies conclude that the induction of apoptosis in lymphoblast cell lines by AA and GLA is due to cell arrest in the G-1 phase and by EPA in Mia-Pa-Ca-2 cells via cell cycle arrest [139;142]. Several studies have also concluded that the induction of apoptosis in CML by PUFA is independent of AA conversion to eicosanoids or free radical generation [143]. Conversely, induction of apoptosis in Mia-Pa-Ca-2 by EPA and

AA has been shown to be prevented with anti-oxidants implicating free radicals [123]. The proposed mechanism for the induction of apoptosis by PUFA covers a broad range and has many disagreements as well as agreements. Thus, the mechanism by which PUFA induce apoptosis in certain cell lines needs much work and delineation.

Apoptosis and Lung Inflammation:

Acute respiratory distress syndrome (ARDS) is an acute inflammatory response characterized by neutrophil accumulation, increased pulmonary capillary permeability, pulmonary edema, increased pulmonary vascular resistance, and progressive hypoxemia [4;150]. ARDS is thought to be a neutrophil-mediated acute inflammatory response. The clearance of neutrophils via apoptosis is critical in the resolution of acute lung inflammation [124]. Apoptotic neutrophils are removed from the lung by alveolar macrophages, thereby limiting tissue damage and reducing inflammation [121;122]. Failure of phagocytosis of apoptotic neutrophils or neutrophil degradation via necrosis will contribute to and exacerbate acute lung injury [122]. The polyunsaturated fatty acids, EPA, GLA, AA, and DHA, are all known to induce apoptosis in certain cell types [123;151]. The ability to modify the eicosanoid profile through enteral nutrition with EPA and GLA is beneficial in improving clinical outcomes in patients with ARDS [117]. These improvements may be a direct result of a decreased number of neutrophils in the lung due to increased neutrophil apoptosis and clearance by macrophages. The ability to increase the number of apoptotic neutrophils in the

lungs of patients with ARDS may lead to new therapies to enhance resolution of inflammation and ultimately a more positive outcome.

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Part 3

Experimental Investigation of the Effects of n-3 and n-6 Polyunsaturated Fatty Acids on Human Promyelocytic HL-60 Cells

I. Eicosapentaenoic Acid and γ-Linolenic Acid Induce Apoptosis in HL-60 Cells[1]

[1]This manuscript has been published with co-authors Brian Daley, Blaine Enderson, and Michael Karlstad in: *Journal of Surgical Research*, 107 (1):145-153, 2002.

Abstract:

Enteral nutrition with eicosapentaenoic acid (EPA; 20:5 n-3) and γ-linolenic acid (GLA; 18:3 n-6) decreased pulmonary inflammation by reducing neutrophil counts and chemotactic factors in bronchoalveolar lavage fluid during acute respiratory distress syndrome (ARDS). We hypothesized that the anti-inflammatory effects of EPA and GLA may be due, in part, to induction of neutrophil apoptosis. The purpose of this study was to determine if EPA and GLA, alone or in combination, trigger apoptotic cell death in human promyelocytic leukemia HL-60 cell line. HL-60 cells were incubated with 10, 20, 50, and 100 μM EPA, GLA or various combinations of EPA and GLA for 2, 4, 8, 12 and 24 hours. Oleic acid (18:1 n-9) was used as a fatty acid control. Flow cytometry using dual-staining with propidium iodide and annexin V-FITC assessed apoptosis, necrosis and viability. Apoptosis was verified by DNA fragmentation as assessed by agarose gel electrophoresis. EPA, GLA, and various combinations of EPA and GLA significantly induced apoptosis and reduced cell viability in HL-60 cells. Viability was significantly reduced to the same extent with the combination of 50

μM EPA\20 μM GLA as compared with 100 μM EPA. These data indicate that EPA and GLA, alone or in combination, reduce cell survival by induction of apoptosis. Thus, induction of apoptosis by select dietary n-3 (EPA) and n-6 (GLA) polyunsaturated fatty acids may be the mechanism of the resolution of pulmonary inflammation in ARDS.

Key Words: Fish oil, Borage Oil, Cell Culture, Polyunsaturated Fatty Acids, ARDS, Inflammation, Pulmonary, Sepsis, Neutrophil, Necrosis

Introduction:

Polyunsaturated fatty acids (PUFA) from fish and borage oil have beneficial dietary effects in variety of health problems such as cardiovascular disease, inflammation, acute respiratory distress syndrome (ARDS) and sepsis [1-3]. Apoptosis has been shown to be induced after exposure to individual PUFA including Eicosapentaenoic acid (EPA; 20:5 n-3), γ-linolenic acid (GLA; 18:4 n-6) and arachidonic acid (AA; 20:4 n-6) in a variety of cell types, *in vitro* [3], however, the combined effects of EPA and GLA on apoptosis is not known.

Eicosapentaenoic acid is found in fish oils and GLA in borage, evening primrose and blackcurrant seed oils [4-6]. We previously showed that enteral nutrition with specialized diets containing EPA and GLA reduced the number of neutrophils in the bronchoalveolar lavage fluid, reduced pulmonary inflammation, and improved clinical outcomes of patients with ARDS [7]. These improvements may be a direct result of fewer neutrophils in the lung due to their reduced

migration and/or increased clearance by alveolar macrophages. Phagocytosis of apoptotic neutrophils by macrophages is an important physiological process in the resolution of pulmonary inflammation [8]. Eicosapentaenoic acid from dietary fish oil is incorporated into and reduces the content of AA in cell membrane phospholipids. Eicosapentaenoic acid is available for enzymatic conversion to 3-series prostaglandins and 5-series leukotrienes [9]. These eicosanoids have anti-inflammatory properties and are considered to be less biologically active than the 2-series prostaglandins and 4-series leukotrienes from AA [10]. γ-Linolenic acid is an n-6 fatty acid and is elongated to dihomo-γ-linolenic acid (DGLA) which can be converted to prostaglandin E_1 or a 15-lipoxygenase metabolite, 15-hydroxyeicosatrienoic acid (15-HETriE), which has been shown to attenuate leukotriene B_4 (LTB_4) formation by inhibiting 5-lipoxygenase [11]. Prostaglandin E_1 has also been shown to indirectly decrease leukotriene formation by the inhibition of 5-lipoxygenase [12]. The activation of neutrophils [4] with lipopolysaccharide will prolong their survival, *in vitro* [13]. This effect can be blocked with LTB_4 receptor antagonists, implying that LTB_4 plays a critical role in neutrophil viability.

We hypothesize that the anti-inflammatory effects of EPA and GLA may be due, in part, to induction of neutrophil apoptosis. The purpose of this study was to determine if EPA and GLA, alone or in combination, trigger apoptotic cell death in the human promyelocytic leukemia HL-60 cell line. We used various physiological concentrations of these fatty acids and at ratios similar to those used in our previous dietary studies, as this may help us understand the

beneficial effects of dietary fish and borage oil in the attenuation of pulmonary inflammation in acute lung injury. We used the HL-60 cell line because it is the preferred immortalized cell line for the study of human neutrophil responses, *in vitro* [14;15].

Materials and Methods:

Cells. The human promyelocytic leukemia cell line HL-60 was obtained from Dr. Robert Hall (Univ. of Tennessee Medical Center, Knoxville, TN). Cells were cultured in RPMI 1640 medium with phenol red supplemented with HEPES (25 mM; BioWhittacker, Walkersville, MD), 10 % heat inactivated fetal bovine serum, 1 % penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO), and 1 % L-glutamine (BioWhittaker, Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % room air in a NAPCO model 5410 incubator (Precession Scientific, Chicago, IL).

Treatments. All chemicals and reagents were from Sigma Chemical (St. Louis, MO), unless otherwise noted. Cell number and viability were assessed using a hemacytometer and trypan blue dye exclusion. Cells were plated in sterile Costar 6 well cell culture plates (Corning, Corning, NY) at a concentration of 300,000 cells/mL. Cells were treated with EPA (100, 50, 20, and 10 μM), GLA (100, 50, 20, and 10 μM), and all possible combinations of the 50, 20, and 10 μM EPA and GLA. All fatty acid treatments were conducted under yellow incandescent light. Methanol was used as vehicle control and oleic acid was

used as fatty acid control. All treatments were conducted in triplicate at 2, 4, 8, 12, and 24-hour time points.

Detection of apoptosis (flow cytometry). Detection of apoptosis by flow cytometry was performed using Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA). Once a desired time point was reached, cells were removed from six well plates and washed with phosphate buffered saline containing 0.1 % bovine serum albumin. Cells were pelleted using an Eppendorf centrifuge, model 5415D (Brinkmann Instuments, Westbury, NY) at 1100 x *g* (3400 rpm) for 2 minutes. Cells were resuspended in 100 μl of 1X annexin V binding buffer (kit). Annexin V-FITC (5 μl) and propidium iodide (10 μl) were added to each treatment in the dark (both from kit). Cells were incubated at room temperature for 15 minutes and kept in the dark. Cell suspensions were raised to a final volume of 500 μl with 1X annexin V binding buffer and placed in 10x75 Falcon culture tubes (Becton Dickinson, Franklin Lakes, NJ) on ice and kept in the dark until flow cytometric analysis. Hydrogen peroxide (10 μM) treated cells were used as a positive control for flow cytometry to properly set the scatter plot gates (i.e. hydrogen peroxide treated cells stained with only annexin V-FITC and hydrogen peroxide treated cells stained with only PI). Flow cytometry was performed using a Becton-Dickenson FACScan flow cytometer and Cell Quest software version 1.2 (Becton-Dickenson, San Jose, CA).

Detection of apoptosis (DNA laddering). Detection of apoptotic DNA laddering was performed as described by McGahon et al., 1995 [16]. Cells at a concentration of 225,000 cell/ml were pelleted using an Eppendorf centrifuge, model 5415D (Brinkmann Instruments, Westbury, NY) at 1100 x *g* (3400 rpm) for 2 minutes. Cells are resuspended in 20 µl lysis buffer (20 mM EDTA (Mallinckrodt, St. Louis, MO), 100 mM Tris, pH 8.0, 0.8 % (w/v) sodium lauryl sarcosine (Bioworld, Dublin, OH)). RNase/T1 cocktail mix (10 µl 1 mg/ml, Ambion, Austin, TX) was added and mixed well by flicking the tip of the tube. This was incubated at 37°C for 75 minutes. Proteinase K (10 µl, Ambion, Austin, TX) was added and incubated for 120 minutes at 50°C. Loading buffer (5 µl) was added to samples and samples were loaded onto a 1.5% agarose gel. Gel was run for 200 minutes at 35 volts in 1X TAE buffer using Bio-Rad mini subcell GT (Bio-Rad, Hercules, CA) and a Pharmacia EPA 250/200 power supply (Pharmacia, Peapack, NJ). Molecular weight marker XIV was used (Roche, Mannheim, Germany). Camptothecin (CAM) at a concentration of 5 µM was used as a positive control for apoptosis. Bands of DNA were visualized using ethidium bromide (Promega, Madison, WI) on a FlourChem 8000 imager (Alpha Innotech, San Leandro, CA).

Statistics. Data are expressed as mean percent ± SEM. Differences in mean percent were determined by a Kruskal-Wallace non-parametric rank test with pairwise group comparisons to determine where the differences lay. Data were

analyzed using CRUNCH version 4.0 statistical package (Crunch Software Corp., Oakland, CA).

Results:

Flow Cytometry: The effects of media, methanol (vehicle) and oleic acid on viability, apoptosis, and necrosis was determined by flow cytometry after a 24-hour incubation in HL-60 cells (Table 3.1.1). The monounsaturated fatty acid, oleic acid, vehicle control (methanol), or cell media alone did not induce apoptosis or necrosis and did not affect viability after 24 hours in HL-60 cells. Oleic acid (100 μM) also did not affect apoptosis, necrosis or viability at 2, 4, and 8 hours (data not shown). Figure 3.1.1 shows that there was no difference on apoptosis, necrosis and viability over wide range of oleic acid concentrations (10-100 μM) when compared with 0 μM oleic acid in HL-60 cells. Since there were no time or concentration dependent effects of oleic acid on apoptosis, necrosis and viability in HL-60 cells, the control data reported in the rest of the study was the average of the 2, 4, 8, 12, and 24-hour incubations over all oleic acid concentrations (n = 120). The average basal rate was, as a proportion of the total cell population, 92.3 % viability, 4.8 % apoptosis, and 2.7 % necrosis.

Flow cytometry scatter plots of 100 μM oleic acid, 50 μM EPA, 100 μM GLA and the combination of 50 μM EPA and 20 μM GLA are shown in figure 3.1.2.

The effects of various concentrations of EPA on apoptosis, necrosis and viability in HL-60 cells are shown in Figure 3.1.3. Eicosapentaenoic acid at a

Table 3.1.1. Effects of controls on apoptosis, viability, and necrosis on HL-60 cells after 24-hour incubation.[1]

	24 Hour Incubation					
	% Apoptosis		% Viability		% Necrosis	
Oleic Acid (100 μM)	4.74[a]	±0.24	90.45[a]	±0.63	2.52[a]	±0.82
Methanol	3.88[a]	±1.57	93.95[a]	±1.90	1.90[a]	±0.76
No Treatment	3.31[a]	±0.77	93.73[a]	±0.50	2.64[a]	±0.28

[a] Means within a column not sharing like superscripts are different ($P<0.05$).

[1] Mean percent of cell population ± SEM as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

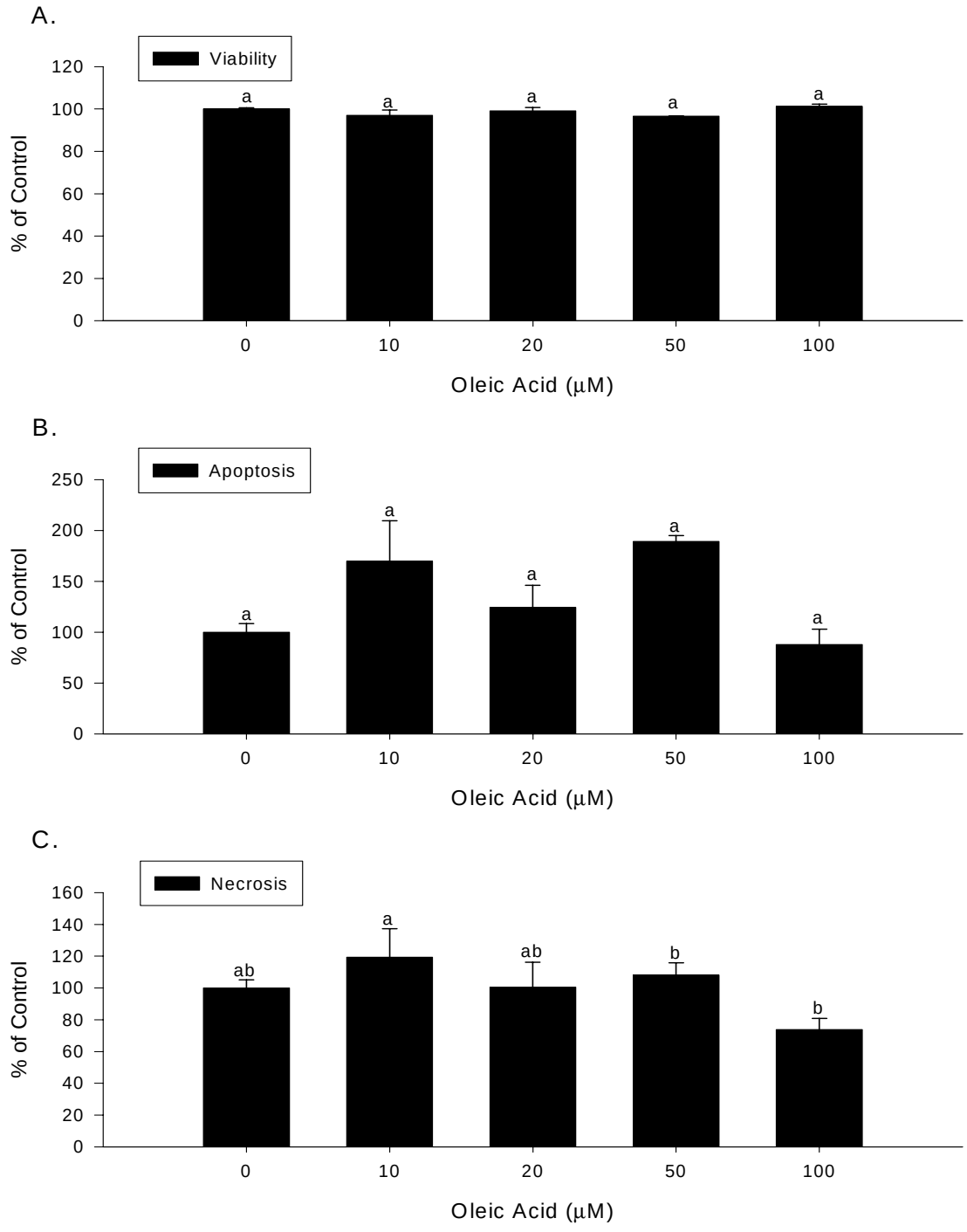
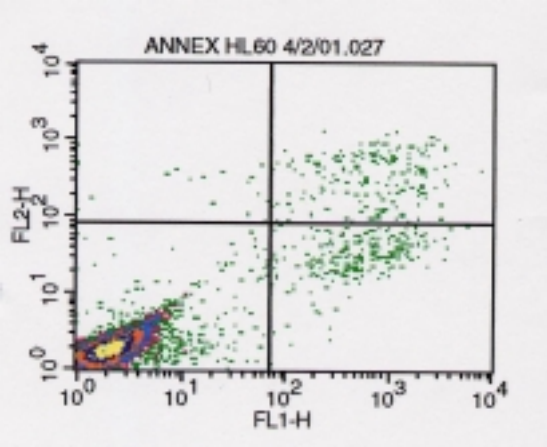


Figure 3.1.1. Effects of oleic acid on viability, apoptosis, and necrosis of HL-60 cells after 12-hour incubation.[1]

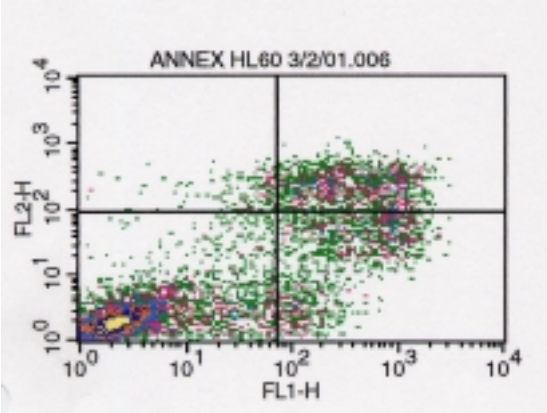
[a,b] For a given variable, values that do not share the same superscript letter are different ($P < 0.05$).

[1]Mean ± SEM of the % viability, apoptosis, and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

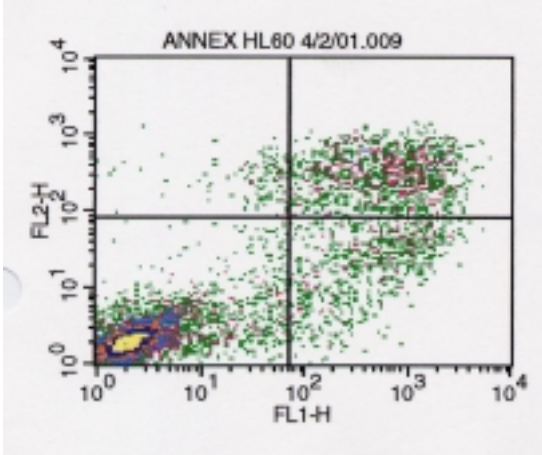
100 μM Oleic Acid



50 μM EPA



100 μM GLA



50 μM EPA 20 μM GLA

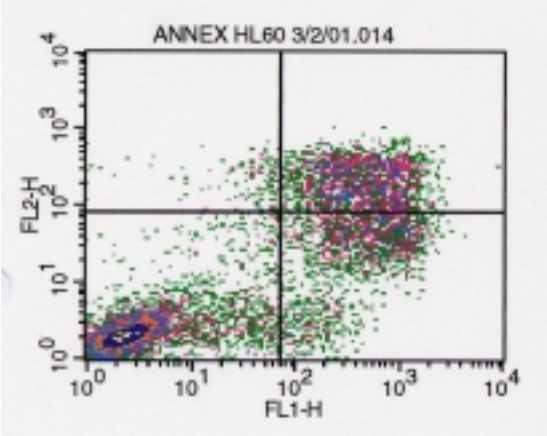


Figure 3.1.2. Flow cytometry scatter plots of 100 μM oleic acid, 50 μM EPA, 100 μM GLA and the combination of 50 μM EPA and 20 μM GLA.

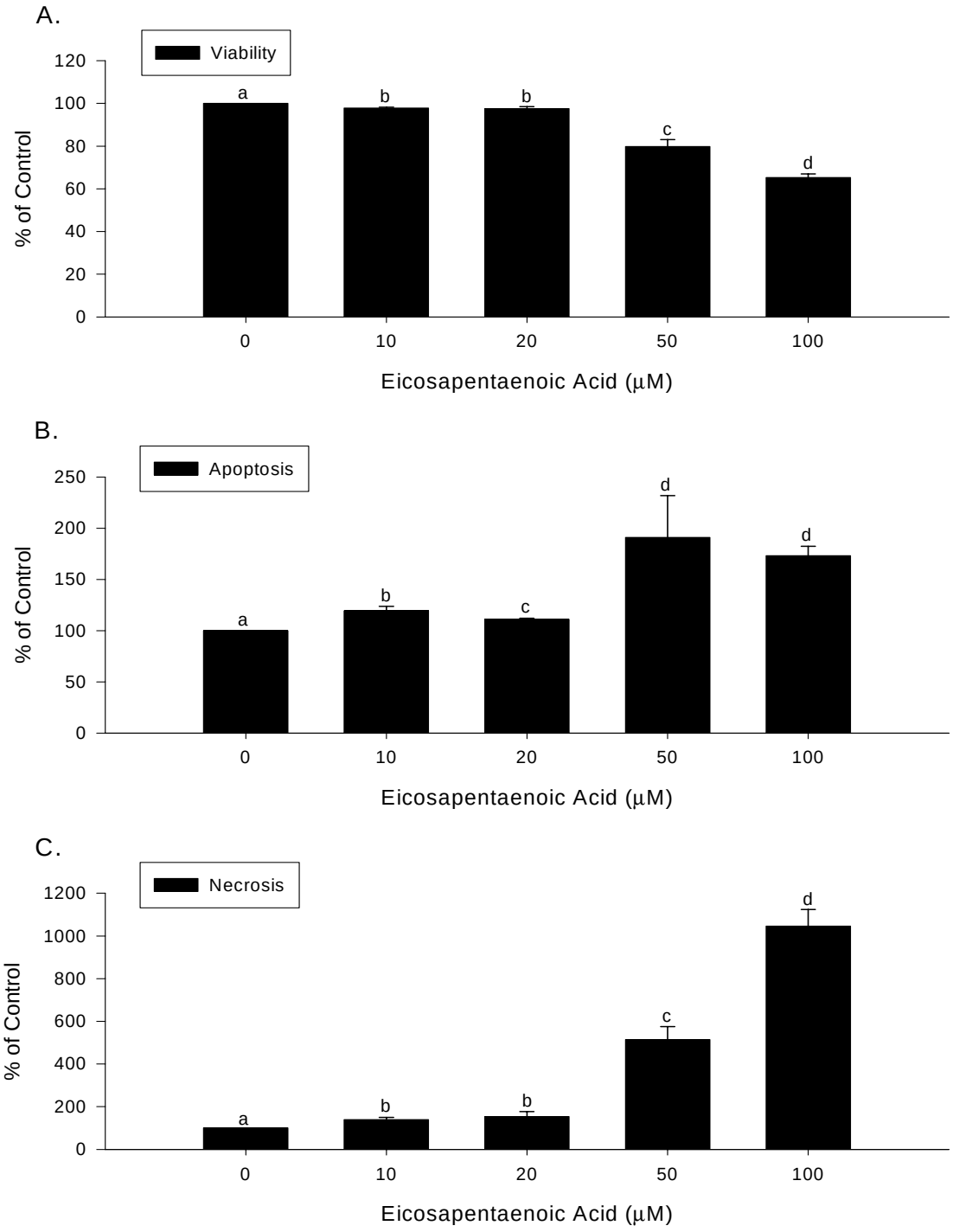


Figure 3.1.3. Effects of eicosapentaenoic acid on viability, apoptosis, and necrosis of HL-60 cells after 12-hour incubation.[1]

[a,b,c,d] For a given variable, values that do not share the same superscript letter are different ($P < 0.05$).

[1]Mean ± SEM of the % of control for viability, apoptosis, and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

concentration of 50 μM induced a significant increase in apoptosis after a 12-hour incubation in HL-60 cells. Viability, apoptosis and necrosis were significantly different from control at 10 μM, 20 μM, 50 μM, and 100 μM EPA. Eicosapentaenoic acid at 100 μM did not cause an additional increase in apoptosis as compared with 50 μM EPA. Secondary necrosis, a feature of end-stage apoptosis, was significantly increased with 50 μM and 100 μM EPA as compared with the lower concentrations of EPA. Secondary necrosis was greatest with 100 μM EPA. These effects of EPA (10-100 μM) on apoptosis and secondary necrosis resulted in a significant corresponding reduction in cell viability. We also noted that duration of the incubation with EPA across all concentrations affected apoptosis, necrosis, and cell viability (data not shown). For example, EPA (100 μM) reduced viability to 27.6% of control, however, this was due to a substantial increase (2211.4%) in secondary necrosis at 24 hours. EPA (10-100 μM) at 2 and 4 hours did not affect apoptosis, necrosis and viability as compared with control.

The effects of various concentrations of GLA on apoptosis, necrosis and viability in HL-60 cells are shown in Figure 3.1.4. GLA exhibits both time and concentration dependent effects on apoptosis, necrosis, and viability in HL-60 cells. There was a significant decrease in viability and increase in secondary necrosis with 100 μM GLA as compared with control and 20 μM GLA. Apoptosis was not induced over the entire range of GLA concentrations as compared with control at 12 hours. We also found that with a 24-hour incubation, viability was

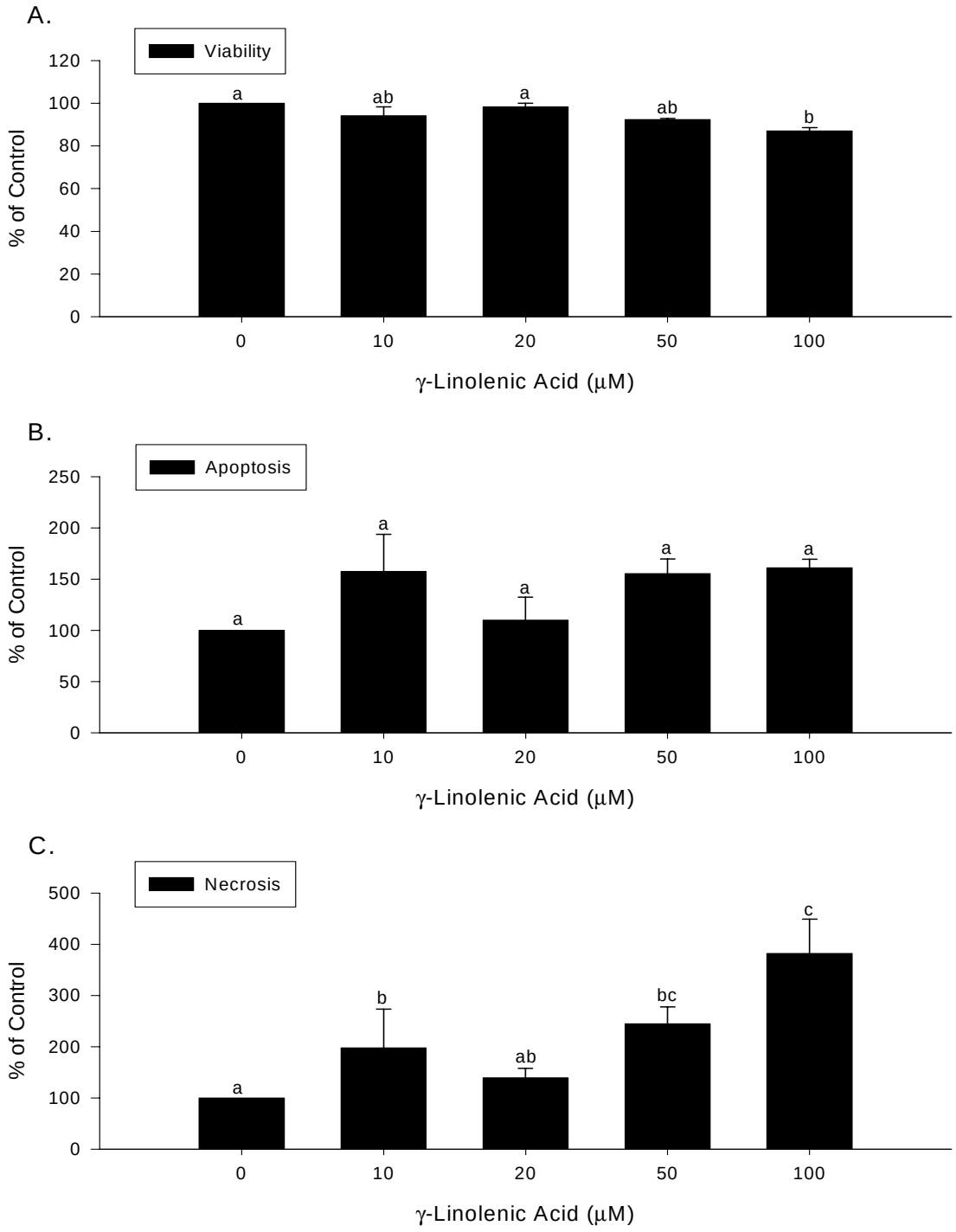


Figure 3.1.4. Effects of gamma-linolenic acid on viability, apoptosis, and necrosis of HL-60 cells after 12-hour incubation.[1]

[a,b,c] For a given variable, values that do not share the same superscript letter are different ($P < 0.05$).

[1]Mean ± SEM of the % of control for viability, apoptosis, and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

significantly decreased and secondary necrosis increased with 50 μM and 100 μM GLA as compared with control, 10 and 20 μM GLA (data not shown). γ-Linolenic acid (0-100 μM) had no effect on viability, apoptosis, and secondary necrosis at the 2, 4, and 8-hour incubation times.

The combined effects of various concentrations of EPA and GLA on apoptosis, necrosis and viability in HL-60 cells are shown in Figure 3.1.5. Apoptosis, necrosis and viability were not significantly different between control and 10 μM EPA combined with either 10, 20, 50 μM GLA or 20 μM EPA combined with either 10, 20, 50 (Figure 3.1.5A). However, 50 μM EPA and 20 μM GLA significantly reduced viability, increased apoptosis and secondary necrosis (Figure 3.1.5B). The combined effects 50 μM EPA and 50 μM GLA and 50 μM EPA and 20 μM GLA significantly reduced viability, increased apoptosis and secondary necrosis. Viability was significantly reduced to the same extent with the combination of 50 μM EPA\20 μM GLA (Figure 3.1.5B) as compared with 100 μM EPA (Figure 3.1.3A).

DNA Laddering. The effect of EPA on DNA fragmentation of HL-60 cells at 12 hour incubation is shown in Figure 3.1.6. There was visible DNA banding of multimers of 180-200 base pairs with 50 and 100 μM EPA at 12 hours. Gamma-linolenic acid, regardless of concentration, did not cause visible DNA banding (data not shown). The effect of various combinations of EPA and GLA on DNA fragmentation of HL-60 cells at the 12-hour incubation is shown in Figure 3.1.7.

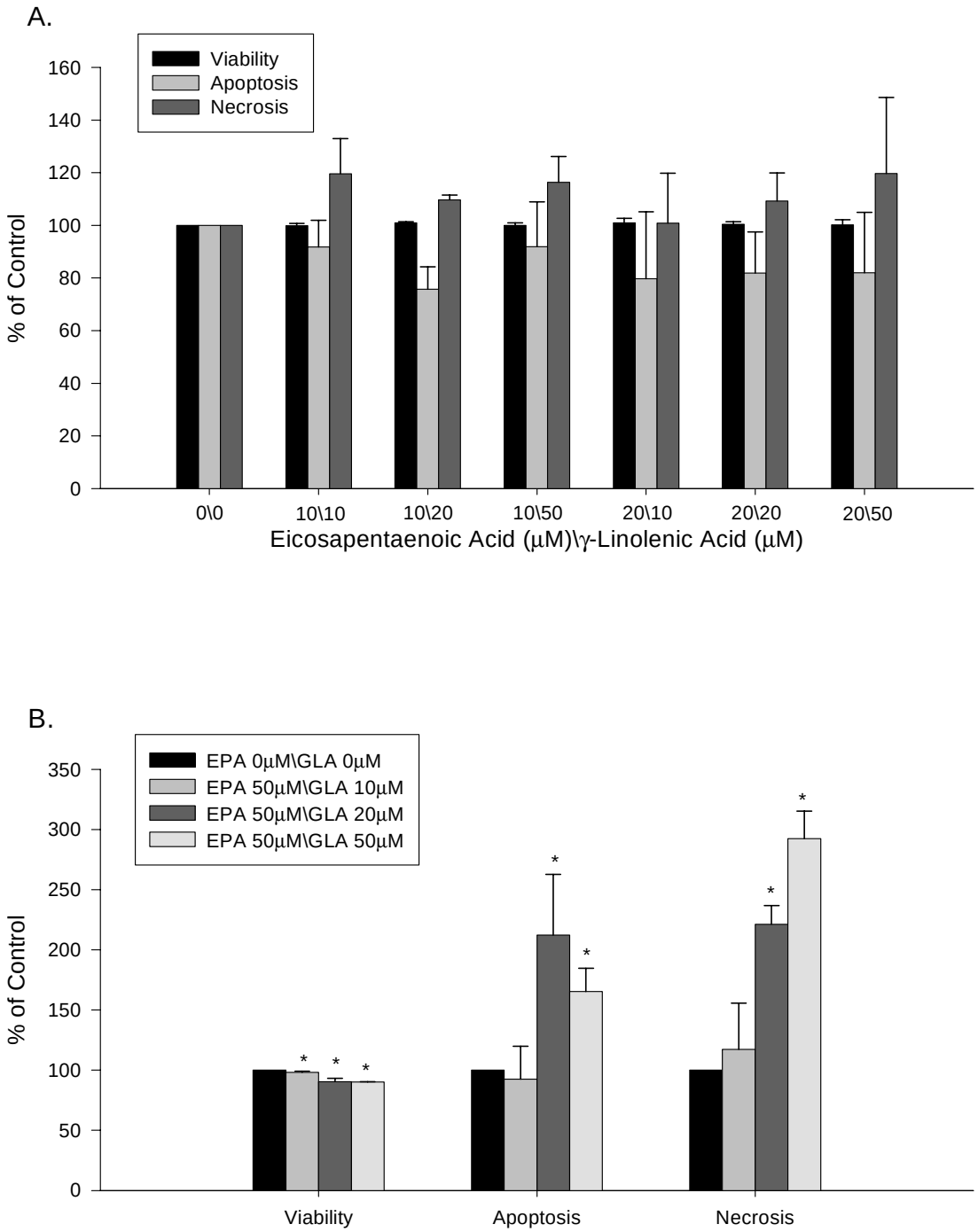


Figure 3.1.5. Effects of various combination of 0, 10, or 20 μM EPA (panel A) and 50 μM EPA (panel B) in the presence or absence of 0, 10, 20, or 50 μM GLA on viability, apoptosis, and necrosis of HL-60 cells after 8-hour incubation.[1]

* For a given variable, value is different from control ($P < 0.05$).

[1]Mean ± SEM of the % of control for apoptosis, viability, and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

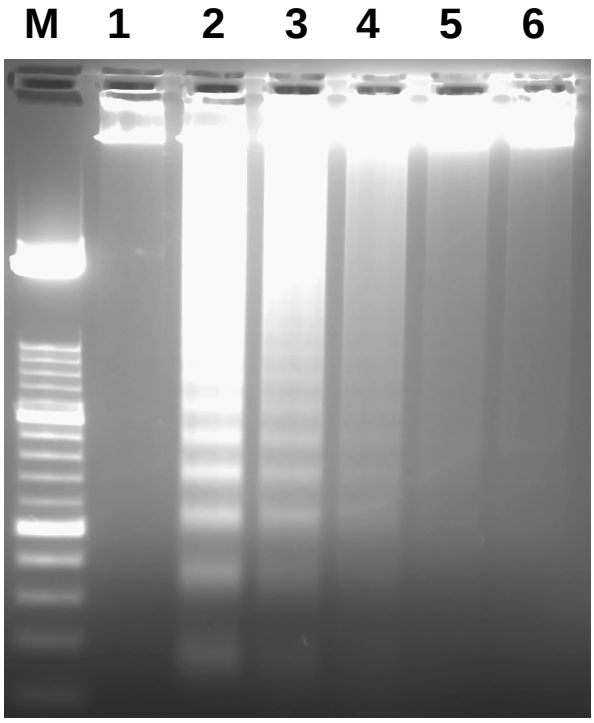


Figure 3.1.6. Effects of eicosapentaenoic acid on whole DNA fragmentation of HL-60 cells after 12-hour incubation.
Lanes: M= marker lane containing a 100 bp ladder of DNA fragments from 2642 bp, 1500-100 bp; 1 = negative control; 2 = Positive control (5 μM CAM); 3 = 100 μM EPA; 4 = 50 μM EPA; 5 = 20 μM EPA; 6 = 10 μM EPA.
Gel: 1.5 % agarose gel.
Running conditions: 200 minutes at 35 volts in 1X TAE buffer.

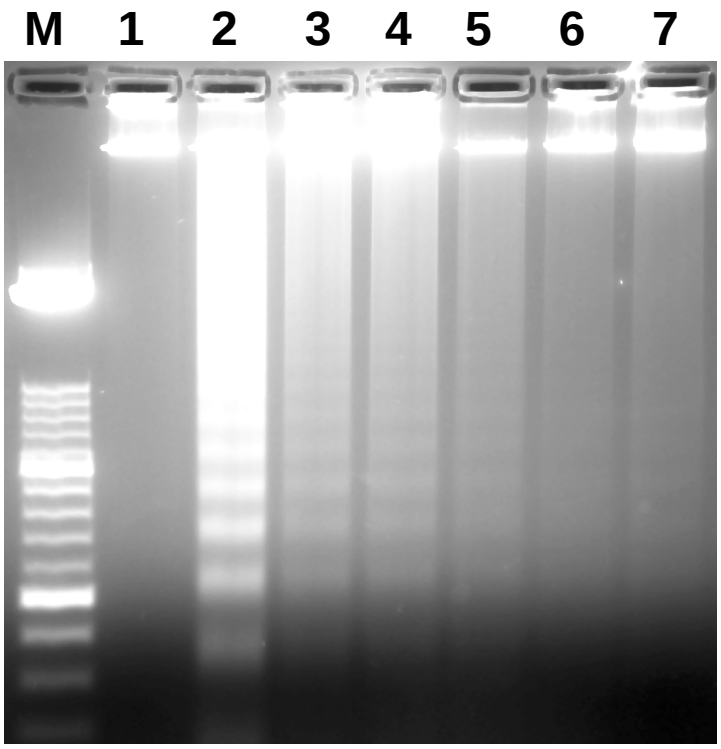


Figure 3.1.7. The effects of various concentrations of EPA and GLA on whole DNA fragmentation of HL-60 cells after 12-hour incubation.
Lanes: M= marker lane containing a 100 bp ladder of DNA fragments from 2642 bp, 1500-100 bp; 1 = negative control; 2 = Positive control (5 μM CAM); 3 = 50 μM EPA\50 μM GLA; 4 = 50 μM EPA\20 μM GLA; 5 = 50 μM EPA\10 μM GLA; 6 = 20 μM EPA\50 μM GLA; 7 = 20 μM EPA\20 μM GLA.
Gel: 1.5 % agarose gel.
Running conditions: 200 minutes at 35 volts in 1X TAE buffer.

The combined effects of 50 μM EPA\50 μM GLA, 50 μM EPA\20 μM GLA, and 50 μM EPA\10 μM GLA produced visible DNA banding of multimers of 180-200 base pairs.

Discussion:

This study shows that eicosapentaenoic acid (EPA) alone or in combination with γ-linolenic acid (GLA) induced apoptosis and secondary necrosis in human promyelocytic HL-60 cells. These results are not likely due to non-specific effects of fatty acids as oleic acid had no effect on apoptosis and secondary necrosis in HL-60 cells (Table 3.1.1). Oleic acid is considered an appropriate fatty acid control as shown in many dietary polyunsaturated fatty acid studies [17;18]. The HL-60 cell line is considered to be the preferred immortalized cell line for the to study human neutrophil responses *in vitro* [14;15]. Eicosapentaenoic acid and GLA are dietary long chain polyunsaturated fatty acids from the n-3 and n-6 series, respectively, and when used in combination in specialized diets have been shown to have anti-inflammatory actions, *in vivo* [7;10]. We also show that various molar ratios of the combination of EPA and GLA had similar effects on reducing cell viability as compared with EPA alone. For example, a total fatty acid load of 70 μM, 50 μM EPA and 20 μM GLA, reduced cell viability as effectively as 100 μM EPA alone. Thus, EPA, GLA, and the combination of EPA and GLA have specific biological activity with respect to a reduction in cell viability in HL-60 cells. Dietary PUFA may have important

therapeutic applications in the clinical management of patients with inflammatory disorders by their ability to modulate cell viability by inducing apoptosis.

HL-60 cells share many functional characteristics with peripheral blood neutrophils, such as containing azurophilic granules, and are, therefore, considered the preferred immortalized cell line model for studying human neutrophil responses *in vitro* [14;15]. Human neutrophils are short lived and will undergo apoptosis in 6-12 hours *in vitro*. HL-60 cells are an immortal cell line and do not spontaneously undergo apoptosis at an accelerated rate. Thus, HL-60 cells are a good substitute to measure the induction of apoptosis by fatty acids and are a good model for peripheral blood neutrophils [15]. Several limitation to the HL-60 cells as a model for neutrophils are that HL-60 cells are a cancer cell line and therefore have different cell cycle regulation than do human neutrophils. Furthermore, HL-60 cells and neutrophils do express the identical gene products. For example, HL-60 cells express Bcl-2 while human neutrophils do not express this protein. Besides the few differences, the HL-60 cell line is still considered the preferred cell line to model human neutrophils.

Apoptosis plays a critical regulatory role in the control of the size of cell populations, normal tissue homeostasis, and resolution of inflammation [8;19]. We performed flow cytometry after dual-staining with Annexin V as a FITC conjugate in combination with propidium iodide (PI) to differentiate between viable, apoptotic and secondary necrotic HL-60 cells. One of the earliest events in apoptosis is the redistribution of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane. During the early phase of apoptosis, the cell

has full membrane integrity [20;21] and Annexin V-FITC is unable to enter the cell and binds only to externalized PS. Since propidium iodide (PI) is too large to pass through the intact cell membrane, it binds to cellular DNA if there is a disruption of cell membrane integrity. Therefore, viable cells will contain neither stain, apoptotic cells stain with Annexin V-FITC and secondary necrotic cells will contain both stains. *In vitro,* secondary necrotic cells have been defined to be in the phase following apoptosis [22;23]. Thus, cells with both stains were most likely due to apoptosis leading to secondary necrosis.

Apoptosis in HL-60 cells followed a dose dependent response to EPA. Substantial loss of cell viability was evident with the higher concentrations of EPA, GLA, and the combination of EPA and GLA at the 12- and 24-hour incubations. Thus, the large decrease in cell viability, particularly at 24-hours, is attributed to apoptosis leading to secondary necrosis. *In vivo*, secondary necrosis occurs when there is a deficiency in the downstream phagocytosis clearance mechanism [8]. If apoptotic cells or apoptotic bodies are not phagocytosed they continue to degrade via secondary necrosis [24]. The translocation of PS to the outer leaflet of the membrane is the means by which phagocytic macrophages recognize apoptotic cells for phagocytosis *in vivo* [25]. Since HL-60 cells are cultured *in vitro* with no phagocytic macrophages in the culture media, they degenerate via secondary necrosis.

It has been reported that HL-60 cells treated with 120 μM EPA reduced cell viability to 44 % of control and apoptosis increased 438 % of control at 24 hours [26]. In the present study, 100 μM EPA at 24 hours reduced viability to 27

% of control and increased apoptosis to 263 % of control. This difference in the extent of apoptosis is most likely due to the fact that the former study used a greater concentration of EPA than the present study. They also reported no detectable DNA fragmentation in HL-60 cells that were treated with EPA using DNA-PI flow cytometry and terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) flow cytometry [26]. However, we were able to detect visible apoptotic DNA fragmentation using gel electrophoresis at 12 hours with 50 and 100 μM EPA treated HL-60 cells.

One of the later stages of apoptosis is DNA fragmentation [24]. The DNA, fragmented between nucleosomes by activated endonucleases, which upon agarose gel electrophoresis give rise to a characteristic DNA ladder pattern of 180-200 base pair multimers [27]. We used camptothecin (CAM) as a positive control for DNA fragmentation because it is a potent inhibitor of topoisomerase I and induces DNA fragmentation in a dose dependent manner [28]. Other studies have reported visible DNA fragmentation of HL-60 cells with 100 μM EPA at 12-, 24-, and 30-hour incubations [3]. We demonstrated positive DNA fragmentation with 50 and 100 μM EPA after 12-hour incubation in HL-60 cells. Although DNA fragmentation using gel electrophoresis was not visible for the GLA treated HL-60 cells, apoptosis was detected by flow cytometry. This may be due to a small percent increase in apoptosis which apparently was not sufficient to be detected by agarose gel electrophoresis.

Acute respiratory distress syndrome (ARDS) is an acute inflammatory process characterized by neutrophil accumulation, increased pulmonary capillary

permeability, pulmonary edema, increased pulmonary vascular resistance, and progressive hypoxemia [29;30]. Enteral nutrition with a diet containing EPA and GLA reduced pulmonary inflammation and improved gas exchange and clinical outcomes in patients with the ARDS [7]. Eicosapentaenoic acid and GLA have been shown to improve lung microvascular permeability, oxygenation, cardiopulmonary function and reduce pro-inflammatory eicosanoid synthesis in animal models of acute lung injury and in human ARDS [7;10]. Additionally there was a substantial reduction in the total number of inflammatory cells and neutrophils in bronchoalveolar lavage fluid [7].

The combination of fish and borage oils are also effective in suppressing synthesis of the pro-inflammatory 2-series prostaglandins and 4-series leukotrienes by alveolar macrophages in endotoxin-induced lung injury. Recently, Barham et al., 2000 [31] showed that dietary EPA and GLA supplementation for three weeks in humans reduced the synthesis of leukotrienes and did not increase the release of arachidonic acid by neutrophils after stimulation with ionophore A23187. They showed that GLA supplementation alone for three weeks increased serum arachidonic acid levels in serum. In contrast, the combination of EPA and GLA did not increase arachidonic acid in serum after three weeks. Thus, the appropriate combination of dietary oils for dietary supplementation may avoid potentially adverse side effects of supplementation with a single dietary oil.

Leukotriene B_4 plays a critical role in neutrophil viability and chemotaxis. Our previous dietary effects may be due to diminished LTB_4-derived chemotactic

activity in bronchoalveolar lavage fluid, and/or a reduction in inflammatory cell survival due to an increase in neutrophil apoptosis. Previous studies have demonstrated that neutrophils activated with lipopolysaccharide, *in vitro*, prolongs neutrophil survival [13]. This prolonged survival can be blocked with LTB_4 receptor antagonists. A reduction of neutrophils in the lung would be beneficial by limiting local tissue injury [32]. Since the elimination of neutrophils is physiologically regulated by apoptosis, the potential to induce apoptosis by dietary lipids would lead to enhanced resolution of inflammation. Thus, induction of neutrophil apoptosis by select dietary PUFA, EPA and GLA, may contribute to improvements in clinical outcomes in ARDS [7].

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The authors gratefully acknowledge R. Andrews for his technical assistance with measurements of flow cytometry.

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II. Role of Downstream Metabolic Processing of Pro-inflammatory Fatty Acids by 5-Lipoxygenase in HL-60 Cell Apoptosis[1]

[1]This manuscript has been published with co-authors Brian Daley, Blaine Enderson, and Michael Karlstad in: *Journal of Trauma,* 54 (1):91-103, 2003.

Abstract:

Pro-inflammatory eicosanoids formed from arachidonic acid (AA) by lipoxygenase (LO) and cyclooxygenase (COX) pathways have been shown to inhibit apoptosis in certain cell types. This study determined if inhibition of LO and COX increased apoptosis in AA treated HL-60 cells, *in vitro*. HL-60 cells were incubated with 50 μM AA and an enzyme inhibitor (1-10 μM) for either COX, LO, 12-LO, and 5-LO for 12 hours. Flow cytometry was used to assess viability, apoptosis, and necrosis. Apoptosis was further assessed using TUNEL and DNA fragmentation. The highest concentration of LO inhibitors, but not COX inhibitors, decreased viability and increased apoptosis and necrosis in the presence of exogenous AA. These results suggest that disruption of the metabolism of AA by LO, in particular 5-LO, decreases cell survival and increases apoptosis. Thus, downstream metabolic processing of AA by LO but not COX plays a critical role in the regulation of HL-60 cell apoptosis.

Key Words: Polyunsaturated Fatty Acids, ARDS, Inflammation, Pulmonary, Neutrophil, Necrosis

Introduction:

Arachidonic acid (AA, 20:4 n-6) is a polyunsaturated fatty acid (PUFA) and a precursor of the pro-inflammatory 2-series prostaglandins and 4-series leukotrienes. The lipoxygenase and cyclooxygenase enzymes convert AA to the 4-series leukotrienes, 2-series prostaglandins, and other bioactive products [1]. Excessive release of AA-derived metabolites from inflammatory cells, including neutrophils and macrophages have been implicated in the development of septic shock, systemic inflammatory response syndrome (SIRS), acute lung injury, and the acute respiratory distress syndrome (ARDS) [2]. The measurement of elevated levels of AA metabolites, including leukotriene B_4 (LTB_4), prostaglandin E_2, and thromboxane B_2, in bronchoalveolar lavage (BAL) fluid in patients with ARDS and/or SIRS has supported their role in the pathophysiology of pulmonary inflammation and ARDS [3;4].

Leukotriene B_4, a product of 5-lipoxygenase (5-LO), is released from activated alveolar macrophages and plays a major role in adherence, chemotaxis, and granulation of polymorphonuclear (PMN) leukocytes, as well as enhancing the ability of PMNs to secrete oxygen free radicals and proteolytic enzymes [5;6]. Previous animal and clinical studies has shown that enteral nutrition supplementation with eicosapentaenoic acid (EPA, 20:5n-3) and γ-linolenic acid (GLA, 18:3 n-6) from fish and borage oil, respectively, reduces pulmonary inflammation as assessed by a reduction in number of neutrophils and levels of LTB_4 in BAL fluid in acute lung injury and ARDS [7]. However, it is not known if the resolution of pulmonary inflammation in those studies was due to a

decrease in neutrophil chemotaxis and/or an increase in apoptosis. The downstream metabolic processing of AA may be critical for prolonged inflammatory cell survival since neutrophil apoptosis is accelerated in the absence of 5-LO products of arachidonic acid [8;9].

It is been shown that diets supplemented with dietary fish and borage oil reduce AA and increase EPA concentrations in tissue and inflammatory cell phospholipids [10;11]. Increased concentrations of EPA in cellular phospholipids can compete with AA for lipoxygenase (LO) and cyclooxygenase (COX), which will reduce pro-inflammatory eicosanoid formation from AA [11;12]. We previously showed that EPA and GLA, and various combinations of EPA and GLA significantly induced apoptosis and reduced cell viability in a time and concentration dependant manner in human promyelocytic HL-60 cells, *in vitro* [13]. A number of studies have shown that AA metabolism is linked to cell proliferation and survival during inflammation and cancer [8;14;15]. The purpose of this study was to determine if the addition of various LO and COX enzyme inhibitors of AA metabolism would affect cellular viability, apoptosis, and necrosis of AA-treated HL-60 cells.

Materials and Methods:

Cells. The human promyelocytic leukemia cell line HL-60 was obtained from American Type Tissue Culture Collection (Manassas, VA). We used the human promylocytic HL-60 cell line because it is considered to the preferred immortalized cell line for the study of human neutrophils responses *in vitro* [16].

Cells were cultured in RPMI 1640 medium with phenol red supplemented with HEPES (25 mM; BioWhittacker, Walkersville, MD), 10 % heat inactivated fetal bovine serum, 1 % penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO), and 1 % L-glutamine (BioWhittacker, Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % room air in a NAPCO model 5410 incubator (Precision Scientific, Chicago, IL).

Treatments. All chemicals and reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Cell number and viability were assessed using a hemacytometer and trypan blue dye exclusion technique. Cells were plated in sterile Costar 6 well cell culture plates (Corning, Corning, NY) at a concentration of 300,000 cells/ml. Cells were treated with 50 μM AA in the presence of an enzyme inhibitor for 12 hours. Compounds used to inhibit enzymes were from Biomol (Plymouth Meeting, PA). Each fatty acid was treated with inhibitors for COX 1 & 2 (ibuprofen [(±)-2-(4-isobutylphenyl) propionic acid]; 1, 5, and 10 μM), 5, 12, 15-LO (NDGA [nodihydroguaiaretic acid]; 1, 5, and 10 μM), 12-LO (baicalein [5,6,7-trihydroxyflavone]; 1, 5, and 10 μM), 5-lipoxygenase (AA-861 [2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone]; 1, 5, and 10 μM), and 5-lipoxygenase activating protein (MK-886; 1, 5, and 10 μM). All fatty acid treatments were conducted under yellow incandescent light. Methanol and DMSO were used as vehicle controls and oleic acid was used as fatty acid control. All treatments were incubated in quadruplicate.

Detection of apoptosis (flow cytometry). Detection of apoptosis by flow cytometry using Annexin V-FITC and propidium iodide stains (BD Pharmingen, San Diego, CA) was performed as previously described by Gillis et al., 2002 [13]. After 12-hour incubation cells were washed once with phosphate buffered saline containing 0.1 % bovine serum albumin. Cells were pelleted using an Eppendorf centrifuge, model 5415D (Brinkmann Instuments, Westbury, NY) at 1100 x *g* (3400 rpm) for 4 minutes. Cells were resuspended in 100 µl of 1X annexin V binding buffer (BD Pharmingen, San Diego, CA). Annexin V-FITC (5 µl) and propidium iodide (10 µl) were added to each treatment in the dark. Cells were incubated at room temperature for 15 minutes and kept in the dark. Cell suspensions were raised to a final volume of 500 µl with 1X annexin V binding buffer and placed in 10x75 Falcon culture tubes (Becton Dickinson, Franklin Lakes, NJ) on ice and kept in the dark until flow cytometric analysis. Flow cytometry was performed using a Becton-Dickenson FACScan flow cytometer and Cell Quest software version 1.2 (Becton-Dickenson, San Jose, CA).

Detection of apoptosis (DNA laddering). Detection of apoptotic DNA laddering was performed as described by McGahon et al., 1995 [17]. Cells at a concentration of 225,000 cell/ml were pelleted using an Eppendorf centrifuge, model 5415D (Brinkmann Instruments, Westbury, NY) at 1100 x *g* (3400 rpm) for 2 minutes. Cells were resuspended in 20 µl lysis buffer (20 mM EDTA (Mallinckrodt, St. Louis, MO), 100 mM Tris, pH 8.0, 0.8 % (w/v) sodium lauryl sarcosine (Bioworld, Dublin, OH)). RNase/T1 cocktail mix (10 µl 1 mg/ml,

Ambion, Austin, TX) was added and mixed well by flicking the tip of the tube. This was incubated at 37°C for 75 minutes. Proteinase K (10 μl, Ambion, Austin, TX) was added and incubated for 120 minutes at 50°C. Loading buffer (5 μl) was added to samples and samples were loaded onto a 1.5% agarose gel. Gel was run for 200 minutes at 35 volts in 1X TAE buffer using Bio-Rad mini subcell GT (Bio-Rad, Hercules, CA) and a Pharmacia EPA 250/200 power supply (Pharmacia, Peapack, NJ). Molecular weight marker XIV was used (Roche, Mannheim, Germany). Bands of DNA were visualized using ethidium bromide (Promega, Madison, WI) on a FluorChem 8000 imager (Alpha Innotech, San Leandro, CA).

Detection of apoptosis (TUNEL). Detection of apoptosis by TUNEL assay was performed using the *in situ* death detection, POD kit (Roche, Mannheim, Germany). Approximately 20,000 cells were cytospun at 500 rpm for 5 minutes (Cytospin3, Shandon, England). Cells were air dried and fixed with Carson solution. Slides were rinsed twice with PBS and were incubated in cytofix/cytoperm solution (100 μl) at 4°C for 20 minutes (Pharmagen, San Diego). Slides were rinsed in perm/wash buffer. Slides were blocked for 10 minutes (100 μl of 0.3% H_2O_2 in methanol) and rinsed in PBS and allowed to dry. 50 μl of TUNEL reaction mixture (kit) was added to slides and incubated in a humidified chamber for 60 minutes at 37°C. Hoechst stain (100 μl) was added and the slides were incubated for 30 minutes at 37°C. Slides were rinsed with PBS and protected with cover slips. Slides were analyzed under an Olympus BX51

fluorescent microscope (Olympus Optical Co., LTD., Japan) and images were captured with a Magmafire SP CCD camera (Optronics, Goleta, CA).

Statistics. Data are expressed as mean percent ± SE. Differences in mean percent were determined by one-way analysis of variance followed by REGWF mean separation to determine statistical significance. $P \leq 0.05$ was considered significant. Data were analyzed using CRUNCH version 4.0 statistical package (Crunch Software Corp., Oakland, CA).

Results:

Oleic acid, methanol (solvent for fatty acids and inhibitors), and DMSO (solvent for baicalein; data not shown) did not induce necrosis or apoptosis and did not affect HL-60 cell viability after 12-hour incubation [13]. Arachidonic acid can be metabolized by a variety of pathways that can be blocked with enzyme inhibitors (Figure 3.2.1.).

The effects of ibuprofen (cyclooxygenase 1 and 2 inhibitor) on viability, apoptosis, and necrosis in AA (50 μM) treated HL-60 cells are shown in Figure 3.2.2. Ibuprofen at a concentration of 5 μM significantly reduced viability and 1 and 5 μM ibuprofen significantly increased necrosis as compared with 0 μM ibuprofen. Viability was not significantly different between 0 μM ibuprofen and 1

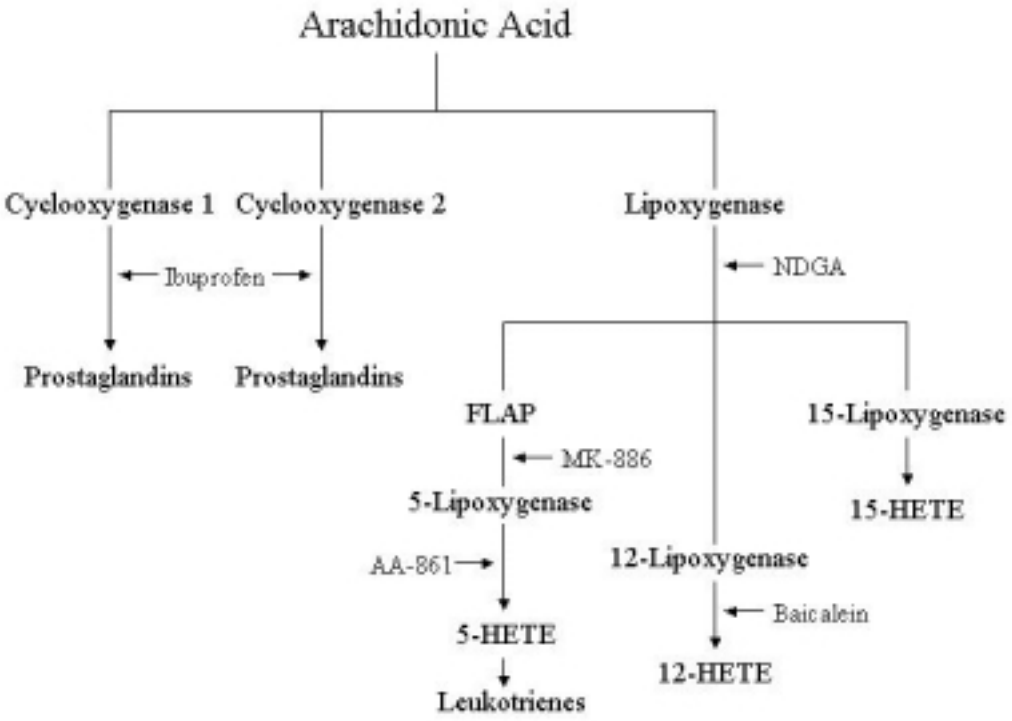

Figure 3.2.1. Schematic of the major pathways involved in arachidonic acid metabolism and inhibitors of the various enzymes involved.

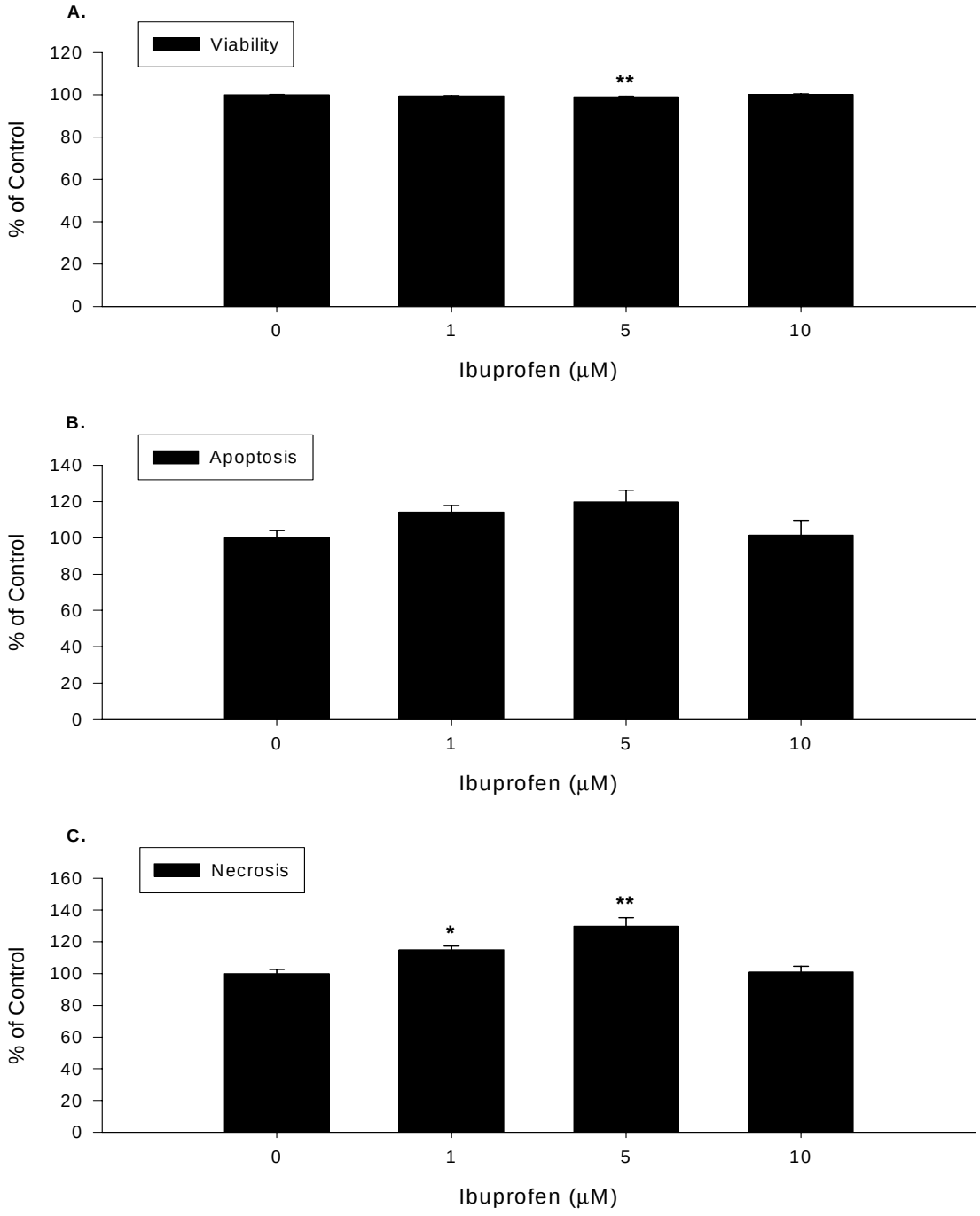

Figure 3.2.2. Effects of ibuprofen (COX 1 and 2 inhibitor) on viability, apoptosis, and necrosis of AA-treated HL-60 cells after 12-hour incubation.[1,2]

* For a given variable, value different from control (P<0.05)

** For a given variable, value different from control (P<0.01)

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM AA.

μM ibuprofen. Apoptosis was not significantly different between 0 μM ibuprofen and 1, 5, and 10 μM ibuprofen. Ibuprofen at a concentration of 10 μM had no effect as compared with 0 μM ibuprofen on HL-60 cell viability, apoptosis, and necrosis after 12-hour incubation.

The effect of various concentrations of NDGA (5, 12, and 15-lipoxygenase inhibitor) on viability, apoptosis, and necrosis in AA (50 μM) treated HL-60 cells are shown in Figure 3.2.3. NDGA at a concentration of 1 μM did not significantly differ from 0 μM NDGA for viability, apoptosis, and necrosis. NDGA at a concentration of 5 μM significantly reduced viability and significantly increased apoptosis when compared with 0 μM NDGA. NDGA at a concentration of 5 μM had no significant effect on necrosis. NDGA at a concentration of 10 μM significantly increased apoptosis and necrosis as well as significantly reduced viability when compared with 0 μM NDGA in AA treated HL-60 cells after 12-hour incubation.

The effects of various concentrations of baicalein (12-lipoxygenase inhibitor) on viability, apoptosis, and necrosis on AA (50 μM) treated HL-60 cells are shown in Figure 3.2.4. Baicalein (5 μM) significantly reduced viability and significantly increased apoptosis and necrosis as compared with 0 μM baicalein. Viability, apoptosis, and necrosis in AA (50 μM) treated HL-60 cells after 12-hour incubation was not significantly different between 0, 1 and 10 μM Baicalein.

The effects of various concentrations of AA-861 (5-lipoxygenase inhibitor) on viability, apoptosis, and necrosis in AA (50 μM) treated HL-60 cells are shown

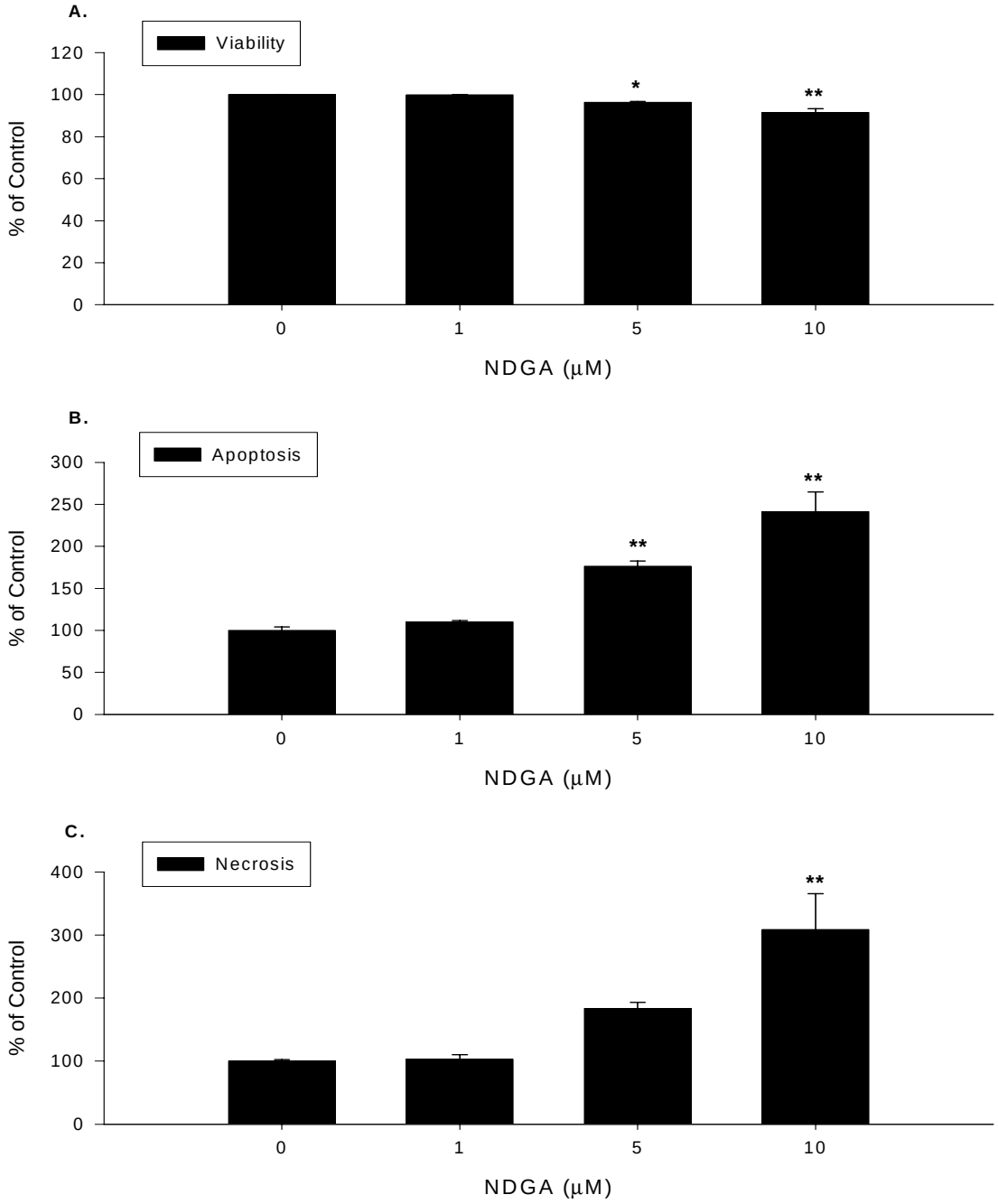

Figure 3.2.3. Effects of NDGA (5-, 12-, and 15-LO inhibitor) on viability, apoptosis, and necrosis of AA-treated HL-60 cells after 12-hour incubation.[1,2]

* For a given variable, value different from control (P<0.05)

** For a given variable, value different from control (P<0.01)

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM AA differ from 0 μM NDGA for viability, apoptosis, and necrosis.

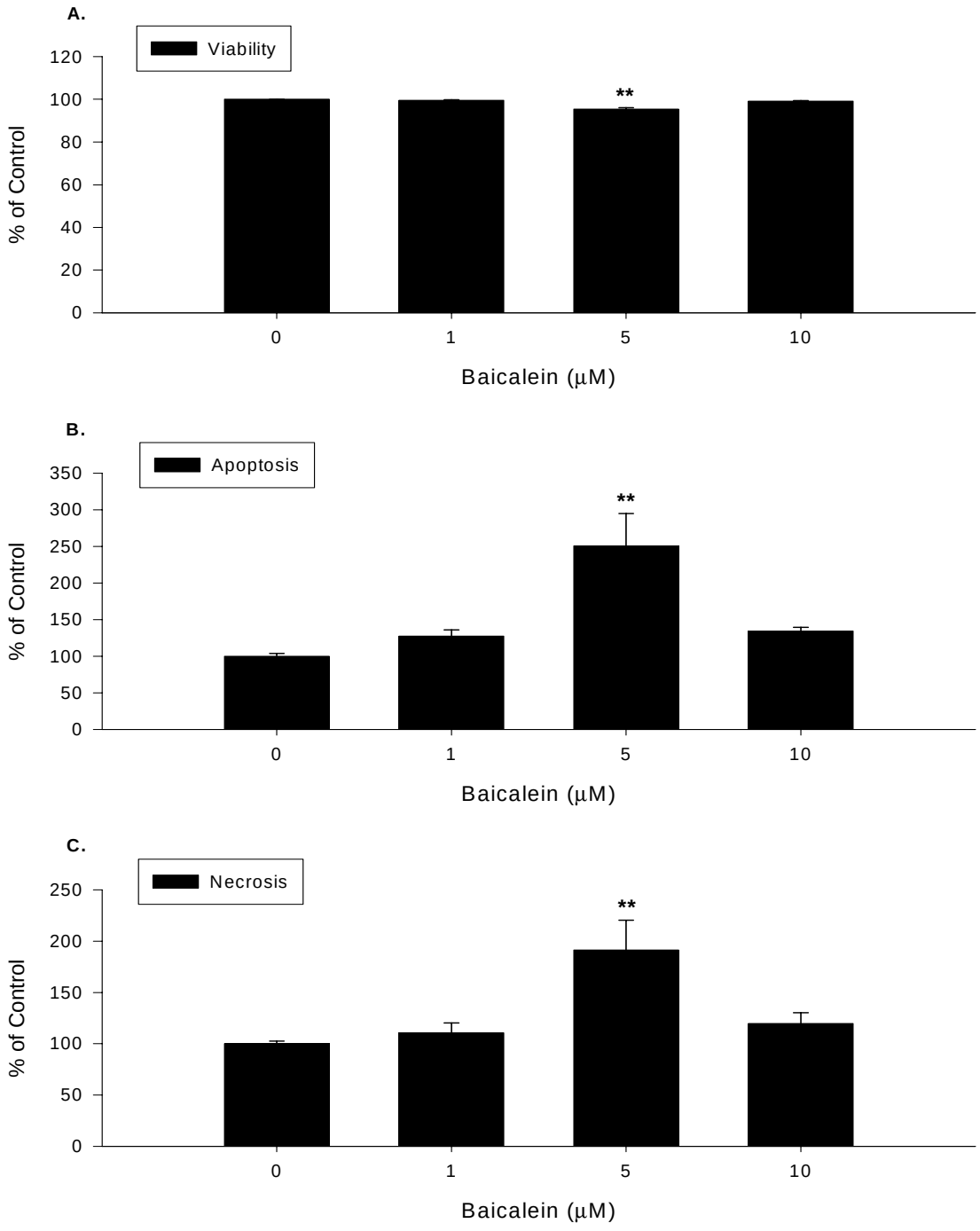

Figure 3.2.4. Effects of baicalein (12-LO inhibitor) on viability, apoptosis, and necrosis of AA-treated HL-60 cells after 12-hour incubation.[1,2]

** For a given variable, value different from control (P<0.01)

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM AA.

in Figure 3.2.5. AA-861 at concentrations of 1, 5 and 10 μM significantly decreased viability as compared with 0 μM AA-861. AA-861 (1, 5, and 10 μM) significantly increased apoptosis and necrosis as compared with 0 μM AA-861 in AA (50 μM) treated HL-60 cells after 12-hour incubation.

The effects of MK-886 (5-lipoxygeanse activating protein inhibitor) on viability, apoptosis, and necrosis in AA (50 μM) treated HL-60 cells are shown in Figure 3.2.6. MK-886 (1 μM) did not significantly affect viability and necrosis as compared with 0 μM MK-886. MK-886 (5 and 10 μM) significantly reduced viability and significantly increased necrosis as compared with 0 μM MK-886. MK-886 (1, 5, and 10 μM) significantly increased apoptosis as compared with 0 μM MK-886 in AA (50 μM) treated HL-60 cells after 12-hour incubation.

The effects of the various enzyme inhibitors tested on whole DNA fragmentation of AA (50 μM) treated HL-60 cells are shown in Figure 3.2.7. There was visible banding of multimers of 180-200 base pairs with 10 μM NDGA, AA-861, and MK-886 at 12 hours. Baicalein (10 μM) caused DNA fragmentation although it is not as obvious as the other inhibitors. Ibuprofen (10 μM) caused no visible DNA fragmentation at 12 hours in AA (50 μM) treated HL-60 cells.

The effects of various enzyme inhibitors tested on a TUNEL assay of AA (50 μM) treated HL-60 cells are shown in Figure 3.2.8. AA (50 μM) treated HL-60 cells incubated with ibuprofen (Ibuprofen treated Panel B) exhibited a positive TUNEL assay as indicated by green fluorescence. AA (50 μM) treated HL-60 cells incubated with NDGA, baicalein, AA-861, or MK-886 (Panels C, D, E, and

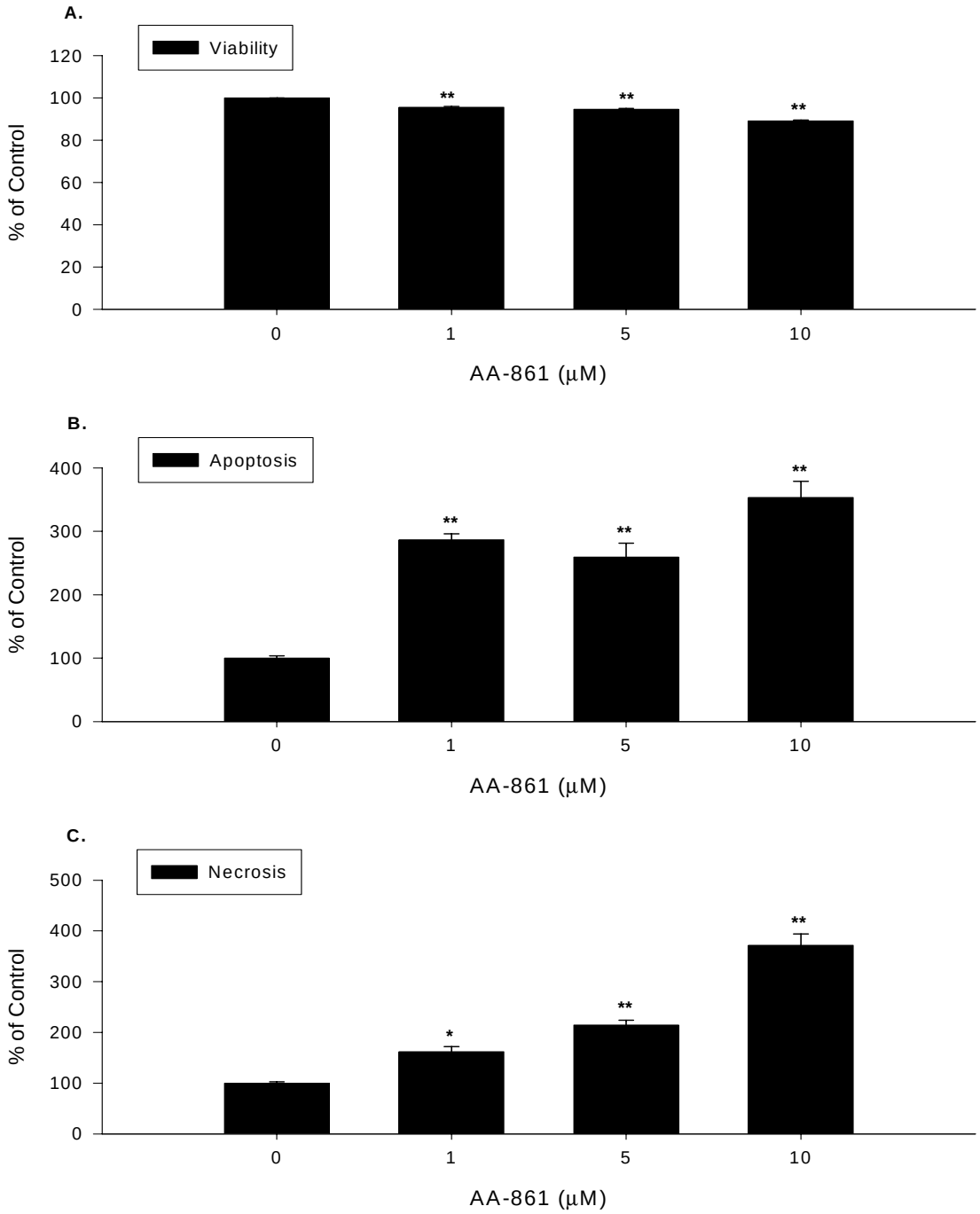

Figure 3.2.5. Effects of AA-861 (5-LO inhibitor) on viability, apoptosis, and necrosis of AA-treated HL-60 cells after 12-hour incubation.[1,2]

* For a given variable, value different from control (P<0.05)

** For a given variable, value different from control (P<0.01)

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM AA.

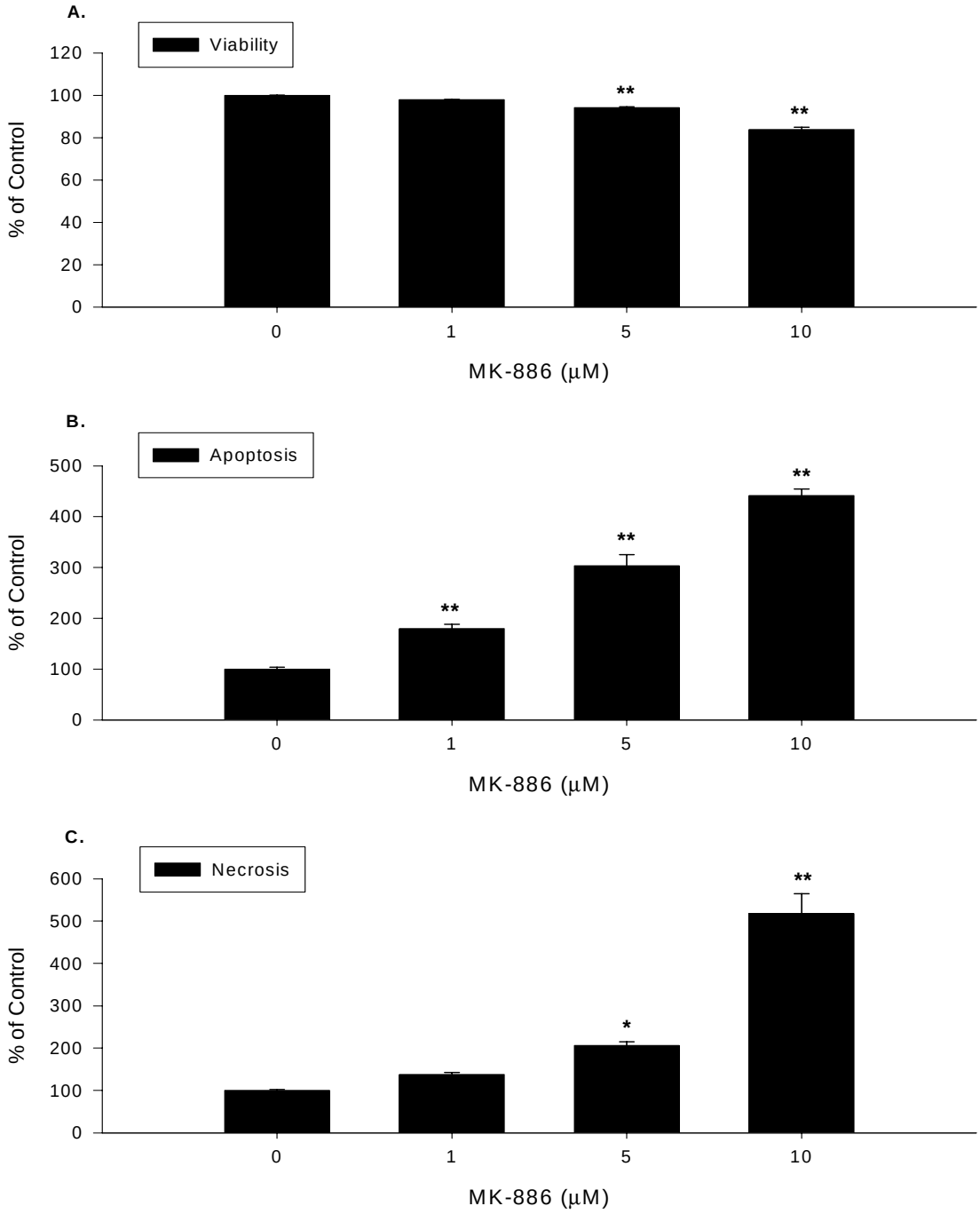

Figure 3.2.6. Effects of MK-886 (FLAP inhibitor) on viability, apoptosis, and necrosis of AA-treated HL-60 cells after 12-hour incubation.[1,2]

* For a given variable, value different from control (P<0.05)

** For a given variable, value different from control (P<0.01)

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM AA.

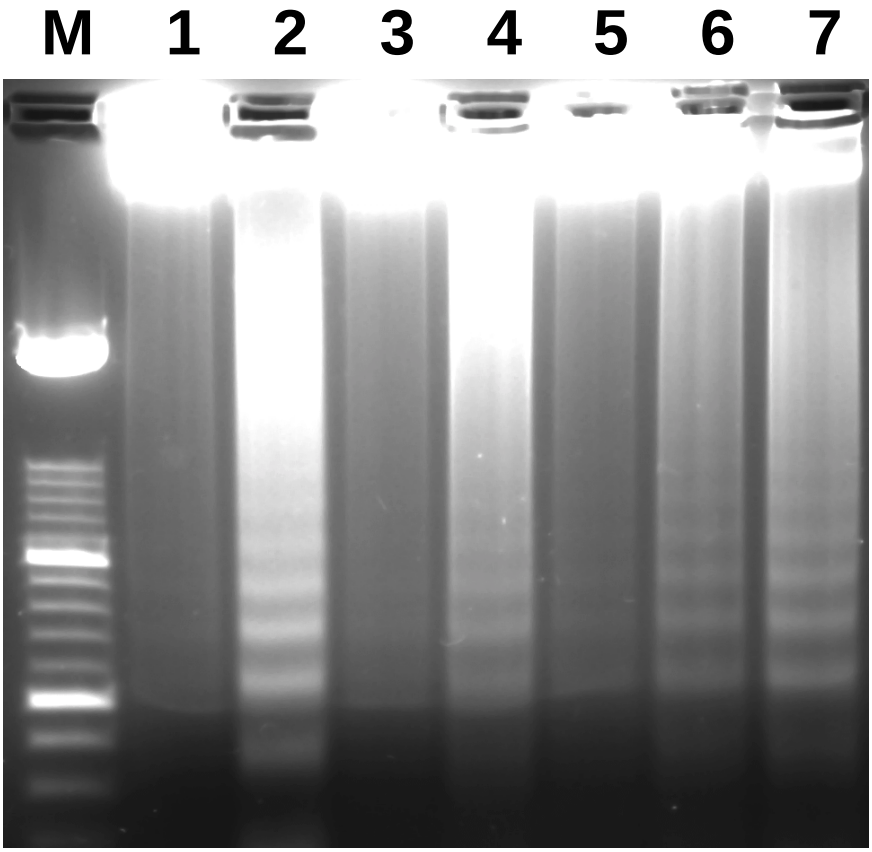

Figure 3.2.7. The effects of various enzyme inhibitors on whole DNA fragmentation of AA-treated HL-60 cells after 12-hour incubation.
Lanes: M= marker lane containing a 100 bp ladder of DNA fragments from 2642 bp, 1500-100 bp; 1 = negative control (50 μM AA); 2 = Positive control (5 μM CAM); 3 = 50 μM AA + 10 μM ibuprofen; 4 = 50 μM AA + 10 μM NDGA; 5 = 50 μM AA + 10 μM baicalein; 6 = 50 μM AA + 10 μM AA-861; 7 = 50 μM AA + 10 μM MK-886.
Gel: 1.5 % agarose gel.
Running conditions: 200 minutes at 35 volts in 1X TAE buffer.

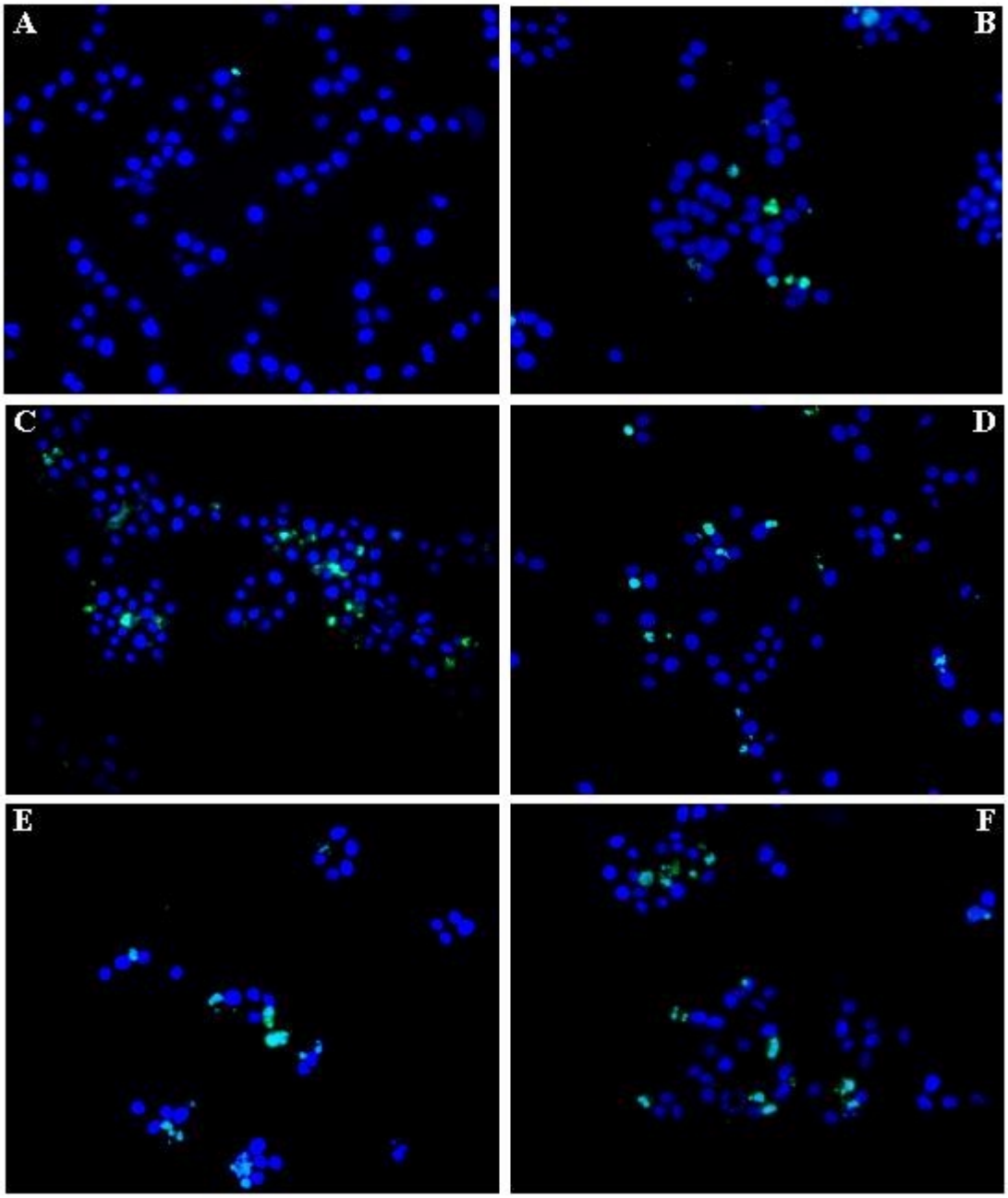

Figure 3.2.8. The effects of various enzyme inhibitors on TUNEL assay of AA-treated HL-60 cells after 12-hour incubation.[1]

A. Negative control; B. 50 μM AA + 10 μM Ibuprofen; C. 50 μM AA + 10 μM NDGA; D. 50 μM AA + 10 μM Baicalein; E. 50 μM AA + 10 μM AA-861; F. 50 μM AA + 10 μM MK-886

[1]Positive TUNEL indicated by green fluorescence; all photos 400x.

F, respectively) all exhibited a positive TUNEL assay indicating that apoptotic cells are present.

Discussion:

This study shows that inhibitors of lipoxygenase enzymes increase apoptosis in HL-60 cells treated with arachidonic acid (AA). The effect of lipoxygenase and cyclooxygenase inhibitors on cellular apoptosis has been shown to both inhibit and induce apoptosis depending on the cell type [9;18-21]. Our results are not likely due to non-specific effects of the enzyme inhibitors since the inhibitors alone had no significant effect on viability, apoptosis, and necrosis in HL-60 cells. Inhibition of the lipoxygenase enzymes, particularly 5-LO, may shunt the metabolism of AA to other pathways involved in the regulation of cellular apoptosis. Thus, it is clear that downstream metabolic processing of AA by lipoxygenases plays a critical role in the regulation of HL-60 cell apoptosis.

Acute respiratory distress syndrome is characterized by increased pulmonary permeability, increased pulmonary vascular resistance, pulmonary edema, progressive hypoxemia, and neutrophil accumulation [22;23]. The ability to alter the fatty acid profile of cellular phospholipids by lowering AA content has been shown to be beneficial in patients with acute inflammation such as that seen in ARDS [7]. Enteral nutrition containing EPA and GLA have been shown to reduce pulmonary inflammation, improve gas exchange and lung microvascular permeability, reduce pro-inflammatory eicosanoid synthesis in animal models of acute lung injury as well as improve clinical outcomes in

patients with ARDS [5;7]. We recently reported that EPA and the combination of EPA and GLA induce apoptosis in a concentration and time dependant manner in HL-60 cells [13]. Feeding fish oil has been shown to increase EPA and reduce AA concentrations in tissue phospholipids [10;11]. It has been shown that diets rich is EPA attenuate some the pro-inflammatory effects of AA [6]. Dietary EPA and GLA supplementation in humans for three weeks reduced the synthesis of leukotrienes and did not increase the release of AA by neutrophils after stimulation with ionophore A23187 [24]. Thus, the use of EPA and GLA in enteral nutrition may induce apoptosis in neutrophils by reducing the amount of AA in the cellular membranes.

HL-60 cells share many functional characteristics with peripheral blood neutrophils such as containing azurophilic granules and are therefore considered to be the preferred immortalized cell line for the study of human neutrophil responses *in vitro* [16;25]. Human neutrophils are short lived and will under go apoptosis in 6-12 hours *in vitro*. HL-60 cells are an immortal cell line and thus are a good model for peripheral blood neutrophils [16].

Apoptosis plays a regulatory role in normal tissue homeostasis, control of cell size populations, and resolution of inflammation [26;27]. In early apoptosis, phosphatidylserine (PS), which is found on the inner leaflet of the cell membrane, translocates to the outer leaflet of the cell membrane while the cell maintains full membrane integrity [28]. We performed flow cytometry after dual-staining of HL-60 cells with annexin V-FITC and propidium iodide (PI) to differentiate between viable, apoptotic, and necrotic cells. During early apoptosis annexin V-FITC is

unable to enter the cell and preferentially binds to externalized PS [29]. Propidium iodide binds to cellular DNA and does not pass through an intact cell membrane. Therefore, viable cells contain neither stain, apoptotic cells stain with only annexin V-FITC, and necrotic cells stain with both annexin V-FITC and PI [30]. Under *in vitro* conditions, secondary necrotic cells have been defined to be in the phase following apoptosis [31;32]. Thus, HL-60 cells that were dually stained were likely due to apoptosis leading to secondary necrosis.

Internucleosomal DNA fragmentation is considered a hallmark of late apoptosis and was used as a second indicator, in conjunction with flow cytometry, to substantiate apoptosis. Cellular DNA is degraded in the internucleosomal regions into double stranded DNA fragments of 180-200 base pairs by endogenous DNAase, giving a characteristic banding upon gel electrophoresis [33;34]. Camptothecin was used as a positive control for DNA fragmentation because it is a potent inhibitor of topoisomerase I and induces characteristic apoptotic DNA fragmentation in a dose dependent manner [30]. Visible DNA fragmentation of 180-200 base pairs was observed for 10 μM NDGA, baicalein, AA-861, and MK-886 treated AA-treated HL-60 cells after 12-hour incubation. No visible DNA fragmentation was observed for ibuprofen treated (10 μM) AA-treated HL-60 cells. These data confirm the observed results of flow cytometry.

Terminal dUTP nick end labeling (TUNEL) is technique to visualize apoptotic DNA fragmentation. Fragmented 3'-OH termini of DNA are modified with terminal deoxynucleotidyl transferase that catalyzes polymerization of

nucleotides to free 3'-OH ends. These strands breaks are fluorescein-labeled and are visualized under a fluorescence microscope [35;36]. TUNEL assay was positive for all five enzyme inhibitors tested. Positive tests correspond with flow cytometry and DNA laddering for NDGA, baicalein, AA-861, and MK-886. The induction of apoptosis with the treatment of ibuprofen was apparent but minimal. A possible explanation for the positive TUNEL assay with ibuprofen may be because TUNEL detects DNA fragmentation over a longer time window as compared with the detection of apoptosis by Annexin V-FITC flow cytometry [37]. Thus, the increase in secondary necrosis with ibuprofen in AA-treated HL-60 cells may have been detected as apoptosis if it were assayed at an earlier time point via flow cytometry. However, the degree of positive TUNEL cells with ibuprofen in AA-treated HL-60 cells was less than that for the other four enzyme inhibitors. Thus, the likelihood that ibuprofen caused a significant increase in apoptosis in AA-treated HL-60 cells is not likely as shown via flow cytometry and DNA laddering.

Apoptosis in HL-60 cells followed a concentration dependent response to the 5-, 12-, 15-lipoxygenase (NDGA), 5-lipoxygenase (AA-861), and 5-lipoxygenase activating protein (MK-886) inhibitors. A substantial increase in HL-60 cell necrosis is observed for the highest concentrations of these enzyme inhibitors. This is attributed to apoptosis leading to secondary necrosis. *In vitro*, secondary necrosis occurs when there is a deficiency in the downstream phagocytosis clearance mechanism [26]. The translocation of PS to the outer leaflet of the cell membrane is one of the means by which phagocytic

macrophages recognize and clear apoptotic cells *in vivo* [38;39]. If apoptotic cells are not cleared they continue to break down via secondary necrosis [33]. Since HL-60 cells were culture *in vitro* with no phagocytic macrophages in the culture media, they degenerated via secondary necrosis.

The two major pathways in the metabolism of AA are the cyclooxygenase (COX) and lipoxygenase (LO) pathways (Figure 3.2.1.) [40]. The cyclooxygenase pathway consists of two primary enzymes, ubiquitously expressed COX 1 and inducible COX 2. Cyclooxygenases 1 & 2 metabolize AA to prostaglandin H_2, which can be converted to other prostanoids, of which many are considered pro-inflammatory [6;41]. Inhibition of COX 1 & 2 had no effect on HL-60 cell apoptosis. However, inhibition of COX 1 & 2 increased necrosis in AA-treated HL-60 cells. It has been shown that inhibition of COX with indomethacin in AA-treated human neutrophils inhibited DNA fragmentation by up to 60% [8]. Indomethacin has been shown to potentiate TNF-α induced apoptosis in HL-60 cells by activation of peroxisome proliferator-activating receptors (PPARs) and not due to inhibition of cyclooxygenase activity [18]. It was also shown that ibuprofen had no effect on TNF-α induced apoptosis in HL-60 cells [18].

The lipoxygenase pathway consists of three primary pathways, 5-LO, 12-LO, and 15-LO (Figure 3.2.1). 5-Lipoxygenase metabolizes AA to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE) which is quickly reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) or leukotriene A_4 (LTA_4). LTA_4 can subsequently be converted to LTB_4 or the cysteinyl leukotrienes (LTC_4, LTD_4,

and LTE_4) [40]. LTB_4 plays a critical role in neutrophil viability and chemotaxis. 5-lipoxygenase activating protein (FLAP) is a membrane associated protein that is critical in AA metabolism by 5-LO [42]. 5-LO and FLAP are highly expressed in PMNs, monocytes, and macrophages [42]. The inhibition of FLAP with MK-886 should produce similar cellular responses to the inhibition of 5-LO by AA-861 in AA-treated HL-60 cells. Inhibition of 5-LO with all concentrations of AA-861 reduced viability and increased apoptosis and necrosis in AA-treated HL-60 cells. Inhibition of FLAP, by MK-886, similarly reduced viability and increased apoptosis and necrosis in AA-treated HL-60 cells. Thus, our results indicate that 5-LO is critical for AA-treated HL-60 cell survival. Lipopolysaccharide (LPS) increases the survival of human neutrophils, *in vitro*, and the inhibition of 5-LO or FLAP decreases survival in LPS treated human neutrophils [43].

12-Lipoxygenase and 15-LO will produce the 12-HETEs and 15-HETEs, respectively (Figure 3.2.1). 15-HETE can be further metabolized to form the lipoxins (lipoxin A_4 and lipoxin B_4), which are considered important compounds in inflammation and immunity [44]. Inhibition of 12-LO with baicalein in AA-treated HL-60 cells reduced cell viability and increased apoptosis and necrosis. However, baicalein at the highest concentration tested (10 μM) had no effect of viability, apoptosis, and necrosis. It is possible that the highest concentration of baicalein was non-specifically binding to other lipoxygenases and possibly other enzymes. Various LO inhibitors have been shown to induce apoptosis in W256 cells which was partially inhibited by the addition of 12-HETE or 15-HETE [45].

We conclude that the lipoxygenase and not the cyclooxygenase pathway were involved in the regulation of apoptosis in AA-treated HL-60 cells. We used several measures of apoptosis including, flow cytometry, DNA fragmentation and TUNEL to substantiate our results and to show that inhibition of the lipoxygenases, in particular 5-LO, increased apoptosis in AA-treated HL-60 cells. The ability to alter the inflammatory cell phospholipid profile to reduce the amount of AA available for metabolism to pro-inflammatory eicosanoids may lead to new therapies that will increase neutrophil apoptosis and provide an injury-limiting pathway in the resolution of pulmonary inflammation in ARDS.

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III. Inhibition of 5-Lipoxygenase Induces Cell Death in Anti-Inflammatory Fatty Acid-Treated HL-60 Cells[1]

[1]This manuscript has submitted for publication with co-authors Brian Daley, Blaine Enderson, and Michael Karlstad.

Abstract:

Enteral nutrition containing eicosapentaenoic (20:5 n-3) and γ-linolenic acid (18:3 n-6) decreases leukotriene B_4 levels and neutrophils in bronchoalveolar lavage fluid of patients and animals with acute respiratory distress syndrome. Reduction in pulmonary inflammation may be due to decreased neutrophil migration and/or survival. We showed that apoptosis increases in eicosapentaenoic/γ-linolenic acid-treated HL-60 cells. However, it is not known if eicosapentaenoic/γ-linolenic acid induced apoptosis involves downstream metabolic products of lipoxygenase and cyclooxygenase enzymes. This study determined the effects of inhibitors of lipoxygenase and cyclooxygenase enzymes on eicosapentaenoic/γ-linolenic acid-treated HL-60 cells. Cells were incubated with 50μM eicosapentaenoic /20μM γ-linolenic acid in the presence of an enzyme inhibitor (1-10μM) for 12 hours. Compounds were used to inhibit cyclooxygenase (ibuprofen), 12-lipoxygenase (baicalein), or 5-lipoxygenase (AA-861). Flow cytometry assessed viability, apoptosis, and necrosis. 5-lipoxygenase inhibition reduced cell viability and increased cell death (apoptosis+necrosis) in eicosapentaenoic/γ-linolenic acid-treated HL-60 cells. Inhibition of cyclooxygenase 1 and 2 and 12-lipoxygenase

had no significant effect on cellular viability and death in eicosapentaenoic/γ-linolenic acid-treated HL-60 cells. Adding leukotriene B_4 counteracted the effect of 5-lipoxygenase inhibition on apoptosis in eicosapentaenoic/γ-linolenic acid-treated HL-60 cells. These data suggest that the processing of eicosapentaenoic and γ-linolenic acid by 5-lipoxygenase is critical to HL-60 cell survival.

Key Words: Fish oil, Apoptosis, Polyunsaturated Fatty Acids, ARDS, Neutrophil

Introduction:

Pro-inflammatory eicosanoids derived from arachidonic acid (AA, 20:4 n-6) play an essential role in the development of septic shock, systemic inflammatory response syndrome, acute lung injury, and acute respiratory distress syndrome (ARDS) [1]. Enteral nutrition with diets containing polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series, eicosapentaenoic acid (EPA, 20:5 n-3) and γ-linolenic acid (GLA, 18:3 n-6), respectively, have been shown to have anti-inflammatory actions *in vivo* [2-5]. Diets supplemented with EPA and GLA reduce arachidonic acid (AA, 20:4 n-6) and increase EPA and dihomo-γ-linolenic acid (DGLA, 20:3 n-6) (GLA is rapidly elongated to DGLA) concentrations in tissue and inflammatory cell membrane phospholipids [6;7] and reduce synthesis of pro-inflammatory eicosanoids in animal models of acute lung injury [3;4]. The ability to alter the fatty acid profile of cellular phospholipids in inflammatory cells has been shown to be beneficial in patients with acute pulmonary inflammation such as ARDS [3]. For example, we showed improved clinical indices of

respiratory function and oxygenation with improved clinical outcomes in patients with ARDS [3;4].

Dietary PUFA of the n-3 and n-6 series may also have important therapeutic applications in the clinical management of patients with inflammatory disorders by modulation of apoptosis and viability of inflammatory cells. Neutrophil clearance by apoptosis is an important physiological mechanism that limits tissue damage and promotes the resolution of inflammation [8]. Leukotriene B_4 (LTB_4), a downstream AA metabolite of 5-lipoxygenase (5-LO), plays a major role in chemotaxis and is critical for prolonged neutrophil survival. [2;4;9;10]. In the absence of the products of 5-LO, cells tend to undergo apoptosis at a faster rate than cells exposed to 5-LO products [11;12]. The downstream metabolites of GLA, PGE_1 and 15-hydroxyeicosatriaenoic acid, have been shown to attenuate LTB_4 production by inhibiting 5-LO [13]. Leukotriene B_5 (LTB_5), a downstream EPA product of 5-LO, is less biologically active than its LTB_4 counterpart [4]. We showed in animal models of acute lung injury that enteral nutrition with EPA and GLA significantly reduced LTB_4 levels in bronchoalveolar lavage fluid (BAL) and decreased pulmonary neutrophil infiltration [4]. Our findings may be due, in part, to diminished numbers of neutrophils in the lung due to a decrease in neutrophil chemotaxis and/or and increase in neutrophil apoptosis.

We recently showed that EPA and the combination of EPA and GLA significantly induced apoptosis and reduced cell viability in human promyelocytic HL-60 cells, *in vitro* [14]. The HL-60 cell line is considered to be the preferred

immortalized cell line for the study of human neutrophil responses *in vitro* [15;16]. HL-60 cells are an immortal human promyelocytic cell line and share many of the same characteristics as human neutrophils such as containing azurophilic granules [15;16]. The purpose of this study was to use this cell line as a model of human neutrophils to determine if the downstream metabolic products of the lipoxygenase (LO) and cyclooxygenase (COX) are involved in the induction of cell death and reduced viability with EPA and GLA in HL-60 cells, *in vitro*. Our results show that the presence of a 5-LO inhibitor caused a further increase in cell death and a substantial reduction in cell viability in EPA and GLA treated-HL-60 cells. Inhibition of 12-LO or cyclooxygenase did not have any effects on EPA and GLA-treated HL-60 cells. Incubation with exogenous LTB_4 counteracted the effects of 5-LO inhibition in EPA and GLA-treated HL-60 cells.

Materials and Methods:

Cells. The human promyelocytic leukemia cell line HL-60 was obtained from American Type Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium with phenol red supplemented with HEPES (25 mM; BioWhittaker, Walkersville, MD), 10 % heat inactivated fetal bovine serum, 1 % penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO), and 1 % L-glutamine (BioWhittaker, Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % room air in a NAPCO model 5410 incubator (Precision Scientific, Chicago, IL).

Treatments. All chemicals and reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Cell number and viability were assessed using a hemacytometer and trypan blue dye exclusion technique. Cells were plated in sterile Costar 6 well cell culture plates (Corning, Corning, NY) at a concentration of 300,000 cells/ml. Cells were treated with 50 μM EPA and 20 μM GLA in the presence of an enzyme inhibitor for 12 hours. Compounds used to inhibit enzymes were from Biomol (Plymouth Meeting, PA). HL-60 cells were treated with inhibitors for COX (ibuprofen [(±)-2-(4-isobutylphenyl) propionic acid]; 1, 5, and 10 μM), 12-LO (baicalein [5,6,7-trihydroxyflavone]; 1, 5, and 10 μM), or 5-LO (AA-861 [2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone]; 1, 5, and 10 μM). HL-60 cells were also incubated with 10 μM AA-861 and were subsequently treated with EPA and GLA as stated above as well as with various concentrations (0.001, 0.01, 0.1, 1.0 μM) of LTB_4 (Cayman Chemical, Ann Arbor, MI). Fatty acid treatments were conducted under yellow incandescent light. Methanol and DMSO were used as vehicle controls and oleic acid was used as fatty acid control. All treatments were conducted in quadruplicate.

Detection of cell viability and cell death (flow cytometry). Detection of cell viability and cell death by flow cytometry was performed using Annexin V-FITC and propidium iodide stains (BD Pharmingen, San Diego, CA) as previously described [14]. Briefly, cells were washed with phosphate buffered saline containing 0.1 % bovine serum albumin. Cells were resuspended in 100 μl of 1X annexin V binding buffer (BD Pharmingen, San Diego, CA). Annexin V-FITC (5

μl) and propidium iodide (10 μl) were added to each treatment in the dark. Cells were incubated at room temperature for 15 minutes and kept in the dark. Cell suspensions were raised to a final volume of 500 μl with 1X annexin V binding buffer. Flow cytometry was performed using a Becton-Dickenson FACScan flow cytometer and Cell Quest software version 1.2 (Becton-Dickenson, San Jose, CA).

Statistics. Data are expressed as mean percent ± SE. Differences in mean percent were determined by one-way analysis of variance followed by REGWF mean separation to determine statistical significance. $P < 0.05$ was considered significant. Data were analyzed using CRUNCH version 4.0 statistical package (Crunch Software Corp., Oakland, CA).

Results:

The metabolism of polyunsaturated fatty acids by lipoxygenase and cyclooxygenase enzymes can be blocked with specific enzyme inhibitors (Figure 3.3.1). Incubation of HL-60 cells in the presence of each inhibitor alone, in the absence of exogenous fatty acids, and with oleic acid did not induce cell death and did not affect cell viability after 12-hour incubation (Table 3.3.1).

The effect of various concentrations of AA-861, a 5-lipoxygenase inhibitor,

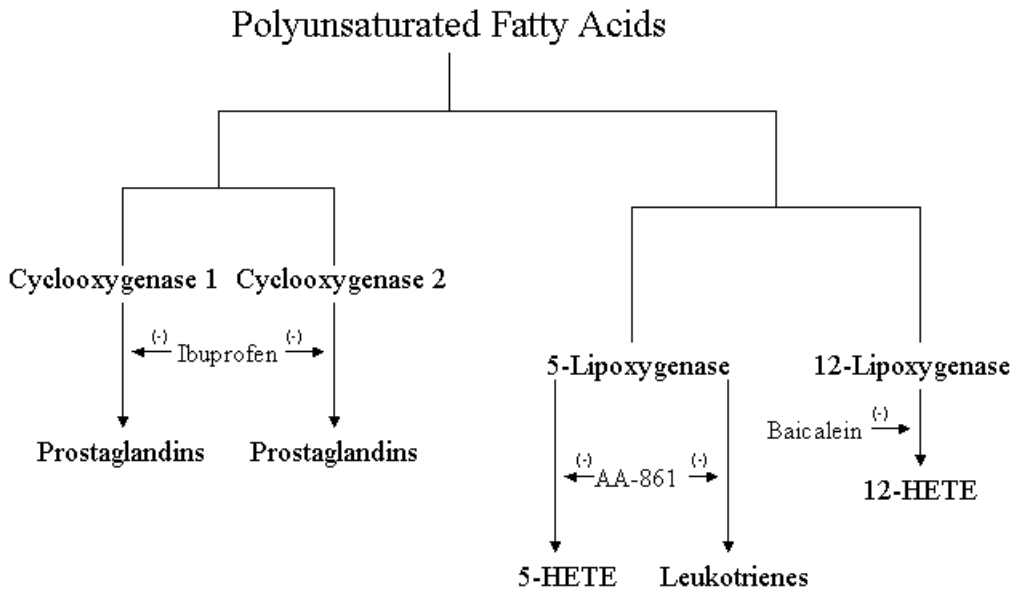


Figure 3.3.1. Major pathways of polyunsaturated fatty acid metabolism and inhibitors of key enzymes.

Table 3.3.1. Effects of controls on cellular viability and death on HL-60 cells after 12-hour incubation.[1]

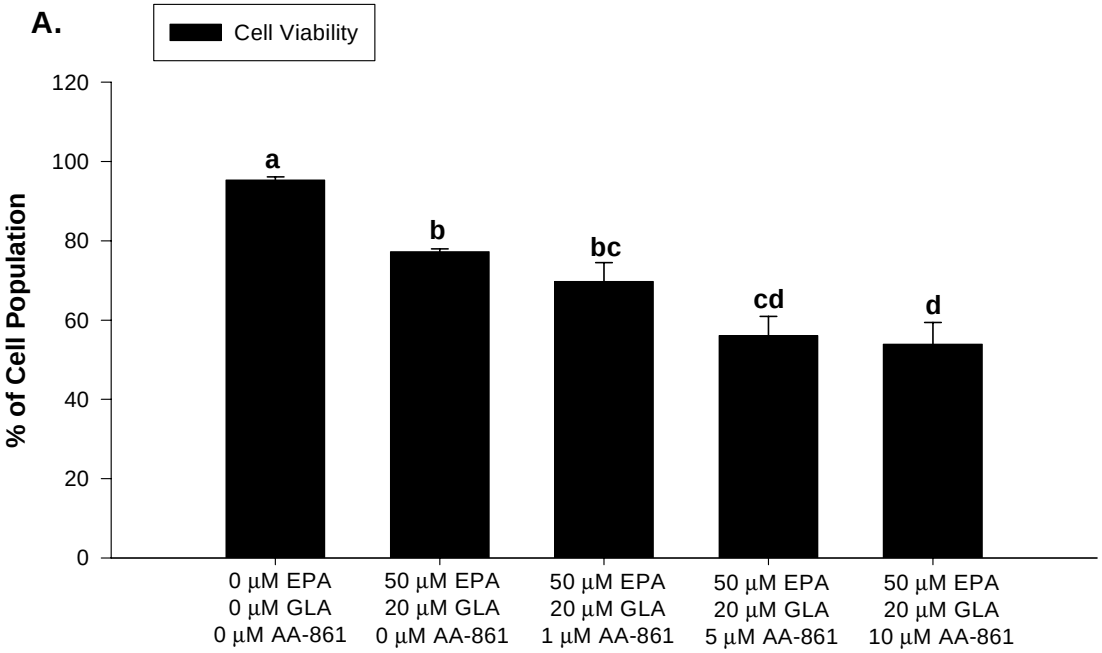
	12-Hour Incubation	
	% Cell Viability	% Cell Death
No Treatment	95.33[a] ± 0.77	4.62[a] ± 0.74
Oleic Acid (70 μM)	92.94[a] ± 1.46	6.93[a] ± 1.40
Ibuprofen (10 μM)	95.72[a] ± 1.02	4.20[a] ± 1.02
Baicalein (10 μM)	95.39[a] ± 1.08	4.56[a] ± 1.06
AA-861 (10 μM)	94.91[a] ± 1.14	5.05[a] ± 1.12

[a] Values within a column sharing the same superscript letter are not different ($P<0.05$).

[1] Mean percent of cell population ± SE as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

on cell viability and cell death in EPA/GLA treated HL-60 cells after 12-hour incubation are shown in Figure 3.3.2A and B. Figure 3.3.2A shows that treatment of HL-60 cells with 50 μM EPA, 20 μM GLA, and 0 μM AA-861 significantly reduced cell viability as compared with no treatment by 1.23 fold. AA-861 at concentrations of 1, 5, and 10 μM significantly reduced cell viability in 50 μM EPA and 20 μM GLA-treated cells as compared with no treatment by 1.37, 1.70, and 1.77 fold, respectively. Additionally, AA-861 at concentrations of 5 and 10 μM further significantly reduced HL-60 cell viability in 50 μM EPA and 20 μM GLA-treated cells as compared with 50 μM EPA, 20 μM GLA, and 0 μM AA-861 treatment by 1.38 and 1.43 fold, respectively. Figure 3.3.2B shows that treatment of HL-60 cells with 50 μM EPA, 20 μM GLA, and 0 μM AA-861 significantly increased cell death as compared with no treatment by 4.84 fold. AA-861 at concentrations of 1, 5, and 10 μM significantly increased cell death in 50 μM EPA and 20 μM GLA-treated cells as compared with no treatment by 6.40, 9.37, and 9.72 fold, respectively. Additionally, AA-861 at concentrations of 5 and 10 μM further significantly increased cell death in 50 μM EPA and 20 μM GLA-treated cells as compared with 50 μM EPA, 20 μM GLA, and 0 μM AA-861 treatment by 1.94 and 2.01 fold, respectively.

The effect of various concentrations of ibuprofen, a cyclooxygenase 1 and 2 inhibitor, on cell viability and cell death in EPA/GLA treated HL-60 cells after 12-hour incubation are shown in Figure 3.3.3A and B. Treatment of HL-60 cells with 50 μM EPA, 20 μM GLA, and 0 μM ibuprofen significantly reduced viability



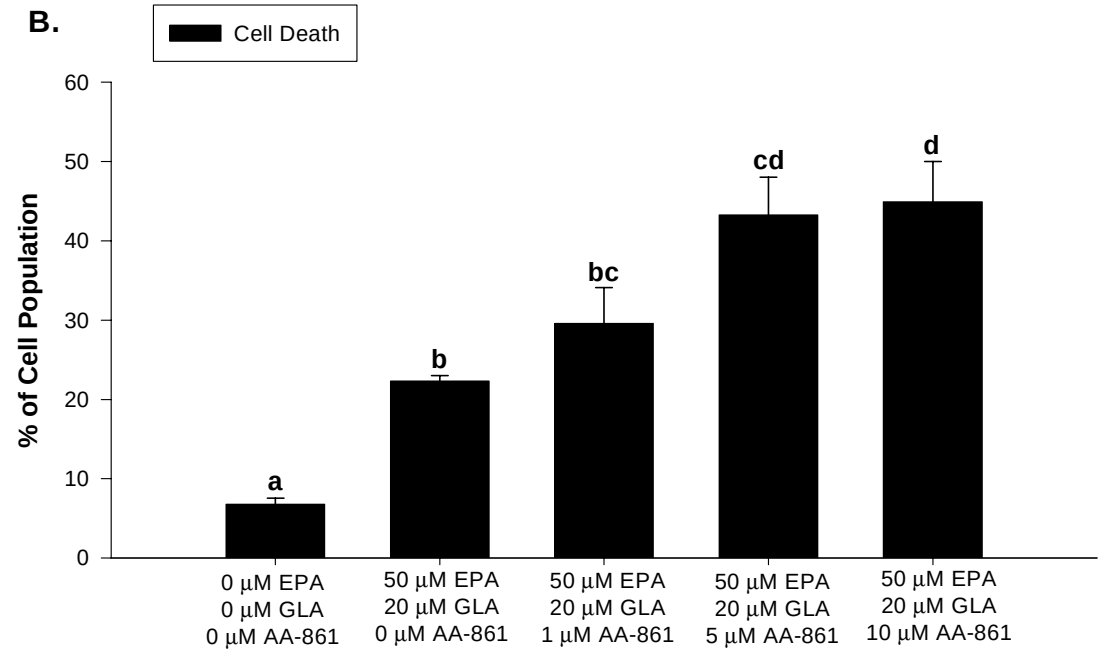


Figure 3.3.2. Effects of AA-861 (5-LO inhibitor) on cell viability and cell death in EPA/GLA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c,d] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SE of the % of cell population for cell viability and cell death as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

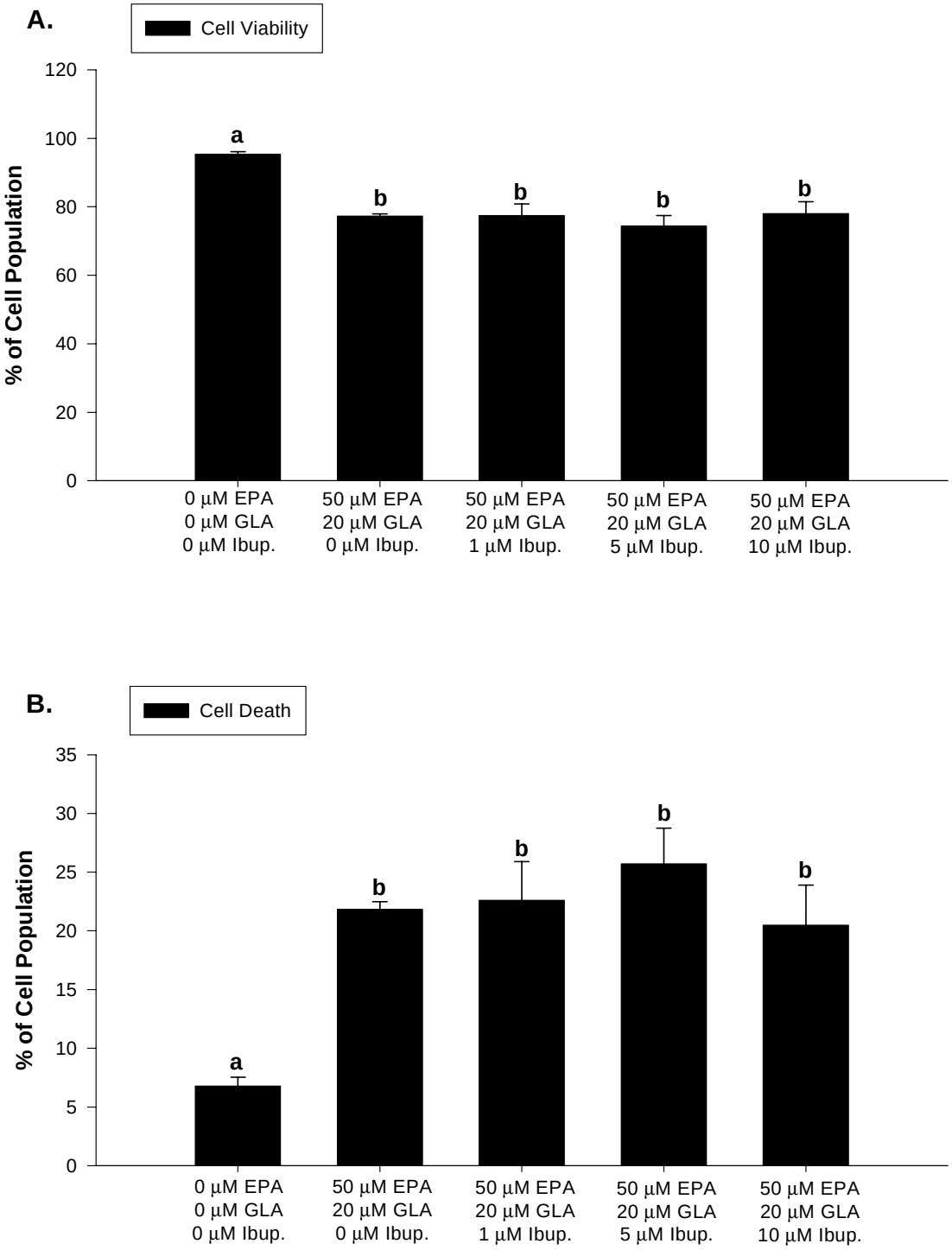


Figure 3.3.3. Effects of ibuprofen (COX 1 and 2 inhibitor) on cell viability and cell death of EPA/GLA-treated HL-60 cells after 12-hour incubation.[1]

[a,b] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SE of the % of cell population for cell viability and cell death as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

and significantly increased cell death as compared with no treatment. However, Ibuprofen (1, 5, and 10 μM), over a wide concentration range, had no significant effect on cell viability and cell death in 50 μM EPA and 20 μM GLA-treated cells as compared with 50 μM EPA, 20 μM GLA, and 0 μM ibuprofen-treated HL-60 cells after a 12-hour incubation.

The effect of various concentrations of baicalein, a 12-lipoxygenase inhibitor, on cell viability and cell death in EPA/GLA treated HL-60 cells after 12-hour incubation are shown in Figure 3.3.4A and B. Treatment of HL-60 cells with 50 μM EPA, 20 μM GLA, and 0 μM baicalein significantly reduced viability and significantly increased cell death as compared with no treatment. However, baicalein (1, 5, and 10 μM) had no significant effect on cell viability and cell death in 50 μM EPA and 20 μM GLA-treated cells as compared with 50 μM EPA, 20 μM GLA, and 0 μM baicalein-treated HL-60 cells after a 12-hour incubation.

The effect of various concentrations LTB_4 and 10 μM AA-861 on cell apoptosis in EPA/GLA treated HL-60 cells after 12-hour incubation is shown in Figure 3.3.5. LTB_4 at a concentration of 1 μM significantly reduced apoptosis in EPA/GLA/AA-861/1μM LTB_4-treated HL-60 cells as compared with EPA/GLA/AA-861/0 μM LTB_4-treated HL-60 cells. Furthermore, this reduction was not different than EPA/GLA-treated HL-60 cells alone. LTB_4 decreased EPA/GLA/AA-861 induced apoptosis in a concentration dependent manner.

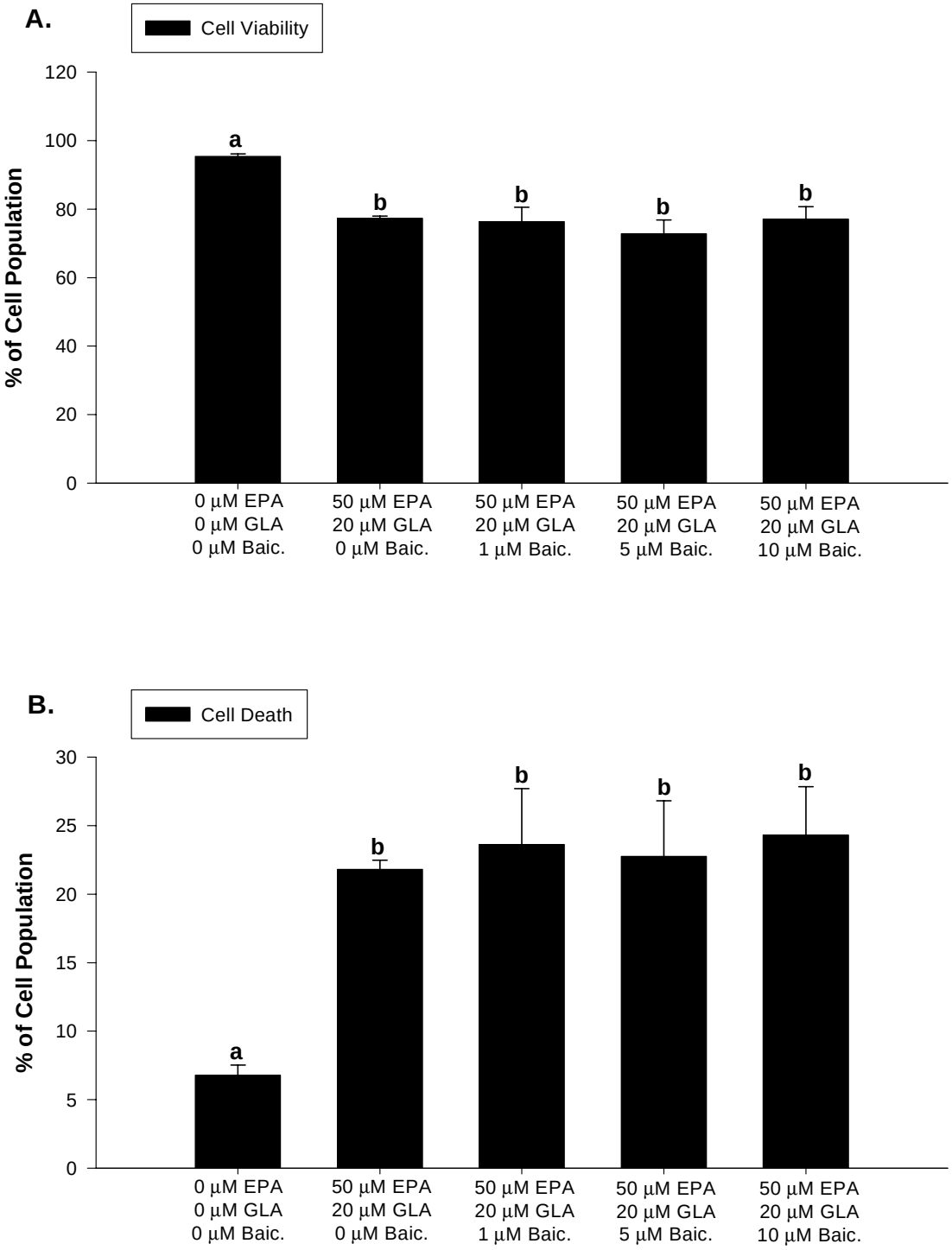


Figure 3.3.4. Effects of baicalein (12-LO inhibitor) on cell viability and cell death of EPA/GLA-treated HL-60 cells after 12-hour incubation.[1]

[a,b] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SE of the % of cell population for cell viability and cell death as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

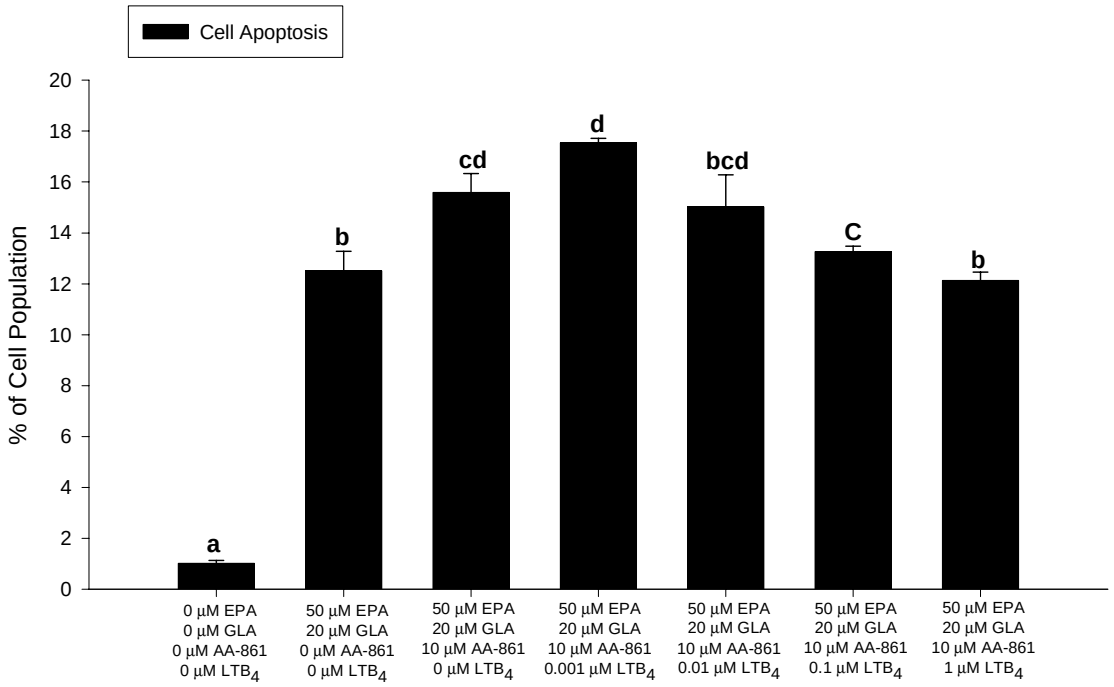


Figure 3.3.5. Effects of various concentrations of LTB_4 on apoptosis of AA-861 and EPA/GLA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c,d] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SEM of the % of cell population for cell apoptosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

Discussion:

We have previously shown that EPA and GLA at the same concentrations used in this study (50 μM EPA and 20 μM GLA) significantly increased apoptosis and secondary necrosis and significantly reduced viability after 12-hour incubation as assessed by flow cytometry and DNA fragmentation in HL-60 cells [14]. This study supports those findings and shows that HL-60 cell death was increased further in the presence of an inhibitor of 5-LO. Thus, there is an additive effect of EPA and GLA with 5-LO inhibition in regards to HL-60 cell death. Conversely, the inhibition of 12-LO or COX 1 and 2 did not affect cell death in EPA/GLA treated HL-60 cells. Incubation with exogenous LTB_4 counteracted the effects of 5-LO inhibition in EPA/GLA treated-HL-60 cells in a concentration dependent manner. These results indicate that LTB_4, a major downstream metabolic product of 5-LO, is important for maintenance of cell survival and suggests that EPA and GLA initiates cell death by a mechanism that likely involves reduced synthesis of LTB_4.

Flow cytometry after dual-staining with annexin V-FITC in combination with propidium iodide (PI) was used to differentiate between viable, apoptotic and secondary necrotic HL-60 cells. Viable cells do not stain with either dye, apoptotic cells with only annexin V-FITC, and secondary necrotic cells with both annexin V-FITC and PI. *In vitro,* secondary necrotic cells have been defined to be in the phase following apoptosis [17;18]. Annexin V-FITC binds to externalized phosphatidylserine, which prior to apoptosis is distributed on the inner leaflet of the cell membrane. *In vivo*, externalized phosphatidylserine is a

physiological marker that macrophages use to recognize and engulf apoptotic cells before they degrade via secondary necrosis [19]. Our system consists of a homogenous cell culture that contains no phagocytic cells. Therefore, apoptotic cells will degrade via secondary necrosis. In this study, HL-60 cells with both stains were taken to be due to apoptosis leading to secondary necrosis and designated cell death. We confirmed, in previous studies, the presence of apoptotic HL-60 cells by the use of several techniques which included flow cytometry, DNA fragmentation, and TUNEL assay [14;20]. We have previously shown that treatment of HL-60 cells with EPA and a combination of EPA and GLA increases apoptosis and secondary necrosis in a time and concentration dependent manner [14]. A reduction in viability and an increase in secondary necrosis were demonstrated at various times between 2 and 24-hour incubation. In addition, apoptosis remained relatively constant between 8 and 24-hours of incubation indicating that cells were undergoing apoptosis at a relatively constant rate and that the increase in secondary necrosis was due to apoptotic cells continuing to degrade via necrosis.

Two of the major pathways of PUFA metabolism are the cyclooxygenase (COX) and lipoxygenase (LO) pathways [21]. The COX pathway consists of two primary enzymes, ubiquitously expressed COX 1 and inducible COX 2. Cyclooxygenase 1 and 2 metabolize AA to prostaglandin H_2, EPA to prostaglandin H_3, and dihomo-γ-linolenic acid (GLA is quickly elongated to dihomo-γ-linolenic acid) to prostaglandin H_1 [2;22]. Inhibition of COX 1 and 2 with ibuprofen had no effect on cell death in EPA/GLA treated HL-60 cells.

Prostaglandin E_1, a GLA metabolite of COX, has been shown attenuate LTB_4 production by inhibiting 5-LO [13]. The lipoxygenase pathway consists of three primary pathways, 5-LO, 12-LO, and 15-LO. 12-LO and 15-LO metabolizes AA to form the 12-HETEs and 15-HETEs, respectively. 15-HETE can be metabolized to form the lipoxins (lipoxin A_4 and lipoxin B_4), which are considered important compounds in inflammation and immunity [23]. We show that inhibition of 12-LO with baicalein had no effect on cell death in EPA/GLA-treated HL-60 cells.

5-lipoxygenase metabolizes AA to form 5-HPETE, which is quickly reduced to 5-HETE or leukotriene A_4 (LTA_4). LTA_4 can subsequently be converted to LTB_4 or the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) [21]. EPA is converted by 5-LO to the 5 series leukotrienes: LTA_5, LTB_5, LTC_5, LTD_5, and LTE_5. Eicosanoids from the 5 series leukotrienes are less biologically active than eicosanoids from the 4 series leukotrienes [4]. LTB_4 plays a critical role in neutrophil chemotaxis and survival [24;25]. 5-LO is highly expressed in PMNs, macrophages, and monocytes [26]. Inhibition of 5-LO metabolism with AA-861 reduced cell viability and increased cell death in EPA/GLA treated HL-60 cells. Thus, our results suggest that 5-LO is critical for EPA/GLA-treated HL-60 cell survival.

We conclude that LTB_4 is important for maintenance of cell survival and suggests that incubation with EPA and GLA initiates cell death by a mechanism that likely involves reduced synthesis of LTB_4. Thus, the ability to alter the fatty acid profile of tissues and inflammatory cells with specialized enteral diets to

increase the amount of EPA and GLA and reduce the AA content in conjunction with 5-LO inhibitory compounds may lead to new therapies that will increase neutrophil apoptosis and provide an injury limiting pathway in the resolution of inflammation in ARDS.

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IV. Regulation of Apoptosis in Eicosapentaenoic Acid Treated HL-60 Cells[1]

[1]This manuscript has submitted for publication with co-authors Brian Daley, Blaine Enderson, Daniel Kestler, and Michael Karlstad.

Abstract:

Neutrophil apoptosis is an important physiological process in the resolution of pulmonary inflammation. Previous studies have shown that eicosapentaenoic acid (EPA; 20:5 n-3) increased the rate of apoptosis in a concentration and time-dependant manner in HL-60 cells. However, it is not known if the EPA induced apoptosis involves the lipoxygenase (LO) and cyclooxygenase (COX) enzymes and/or the downstream metabolic products of these enzymes. Thus, the objective of this study was to determine the effect of inhibitors of LO and COX enzymes on apoptosis, viability, and necrosis in EPA-treated HL-60 cells. Cells were incubated with 50 μM EPA in the presence of an enzyme inhibitor (1-10 μM) for 12 hours. Compounds were used to inhibit either COX 1 and 2 (ibuprofen), 5-, 12-, 15-LO (NDGA), 12-LO (baicalein), 5-LO (AA-861), and 5-LO activating protein (MK-886). Eicosanoid (0.001-1.0 μM) add back experiments were also conducted; LTB_4 and 5-HETE with 5-LO inhibition and 12-HETE with 12-LO inhibition. Flow cytometry, TUNEL assay, and caspase-3 western blotting were used to assess apoptosis. Inhibition of COX 1 and 2 had no effect on apoptosis. Inhibition of 5-LO and 12-LO significantly increased apoptosis in EPA-treated HL-60 cells. Furthermore, addition of LTB_4 reduced apoptosis to levels significantly

lower than EPA-treated HL-60 cells alone. 5-HETE and 12-HETE also lowered apoptosis to control levels. These data indicate that inhibition of LO, particularly 5-LO, increased apoptosis in EPA-treated HL-60 cells. Furthermore, this study clearly demonstrated that the products of the LO enzymes, particularly LTB_4, are critical in the regulation of apoptosis in EPA-treated HL-60 cells.

Key Words: Acute Respiratory Distress Syndrome (ARDS), Inflammation, Neutrophil, Pulmonary, Fish Oil

Introduction:

The acute respiratory distress syndrome (ARDS) is characterized by an increase in neutrophil accumulation in the airways and alveoli [1]. Pulmonary injury is caused by the release of oxygen free radicals by activated neutrophils in ARDS [2]. The clearance of apoptotic neutrophils by alveolar macrophages limits tissue damage [3] and may play a critical physiologic role in the resolution of pulmonary inflammation in acute lung injury and ARDS [4]. Serial bronchoalveolar lavage data demonstrates a progressive decrease in lung neutrophils as ARDS patients improve and are weaned from mechanical ventilation [1]. These findings demonstrate a central role for neutrophils in the pathogenesis of ARDS and suggest that interventions should be aimed at regulating the rate of neutrophil apoptosis to resolve the inflammatory response in the lung.

Apoptosis can be induced in many cell types, including neutrophils, by *in vitro* exposure to various n-3 and n-6 polyunsaturated fatty acids (PUFA)

including eicosapentaenoic acid (EPA, 20:5 n-3), γ-linolenic acid (GLA, 18:4 n-6), and arachidonic acid (AA, 20:4 n-6) [5-8]. Enteral nutrition with EPA and GLA attenuate some of the pro-inflammatory effects of AA-derived eicosanoids [3;9-12]. Several enteral nutrition studies have demonstrated that EPA and GLA decreased the number of neutrophils and LTB_4 levels in the bronchoalveolar lavage fluid of both patients and animals with ARDS [11;12]. EPA and GLA also reduced pulmonary inflammation and improved clinical outcomes in patients with ARDS [12]. These improvements may be due to fewer neutrophils in the lung, which may be a direct result of neutrophil apoptosis induced by changes in the leukotriene profile in the lung.

EPA is incorporated into and reduces the content of AA in cell membrane phospholipids [13;14] and competes with AA for enzymes that produce pro-inflammatory eicosanoids from AA [14;15]. One of the most potent chemoattractants for neutrophils is the AA-derived product of 5-lipoxygenase, leukotriene (LT) B_4. LTB_4 also enhances the ability of neutrophils to secrete oxygen free radicals and proteolytic enzymes [11;16]. LTB_4 has also been associated with prolonged inflammatory cell survival. In the absence of AA derived 5-LO products, cells undergo apoptosis at a faster rate than cells exposed to AA-derived 5-LO products [6;17]. Neutrophils exposed to lipopolysaccharide, *in vitro,* have a prolonged survival which can blocked with LTB_4 receptor antagonists [18]. Parenteral nutrition with EPA has been shown to increase leukocyte membrane and plasma phospholipid content of EPA and

leukocyte production of LTB_5 in postoperative trauma patients [19]. LTB_5, an EPA derived product of 5-lipoxygenase, is less biologically active than LTB_4 [11].

Human promyelocytic HL-60 cells share many functional characteristics with peripheral blood neutrophils, such as containing azurophilic granules, and are therefore considered to be the preferred immortalized cell line for the study of human neutrophil responses *in vitro* [20;21]. We previously showed that the rate of apoptosis increased in a time and concentration-dependent manner in EPA-treated HL-60 cells [7]. Furthermore, studies have demonstrated that the rate of apoptosis in significantly increased with inhibition of lipoxygenase enzymes in AA-treated HL-60 cells [8]. However, it is not known if EPA-induced apoptosis involves the lipoxygenase (LO) and cyclooxygenase (COX) enzymes and/or the downstream metabolic products of these enzymes. Thus, the objective of this study was to determine the effect of inhibitors of LO and COX enzymes on apoptosis, viability, and necrosis in EPA-treated HL-60 cells. In addition, eicosanoid add-back experiments were conducted to investigate products of the lipoxygenase enzymes involved in the regulation of apoptosis in EPA-treated HL-60 cells.

Materials and Methods:

Cells. The human promyelocytic leukemia cell line HL-60 was obtained from American Type Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium with phenol red supplemented with HEPES (25 mM; BioWhittaker, Walkersville, MD), 10 % heat inactivated fetal bovine serum, 1 %

penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO), and 1 % L-glutamine (BioWhittaker, Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % room air in a NAPCO model 5410 incubator (Precision Scientific, Chicago, IL).

Treatments. All chemicals and reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Cell number and viability were assessed using a hemacytometer and trypan blue dye exclusion technique. Cells were plated in sterile Costar six well cell culture plates (Corning, Corning, NY) at a concentration of 300,000 cells/ml. Cells were treated with 50 μM EPA in the presence of an enzyme inhibitor for 12 hours. 50 μM EPA and a 12-hour incubation were chosen based on optimal responses in previous experiments using HL-60 cells [7]. Compounds used to inhibit enzymes were all from Biomol (Plymouth Meeting, PA). Each fatty acid was treated with inhibitors for COX 1 and 2 (ibuprofen [(±)-2-(4-isobutylphenyl) propionic acid]; 1, 5, and 10 μM), 5-, 12-, and 15-LO (NDGA [nodihydroguaiaretic acid]; 1, 5, and 10 μM), 12-LO (baicalein [5,6,7-trihydroxyflavone]; 1, 5, and 10 μM), 5-LO (AA-861 [2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone]; 5, 10, and 20 μM), and 5-lipoxygeanse activating protein (MK-866; 1, 5, and 10 μM). All fatty acid treatments were conducted under yellow incandescent light. Methanol and DMSO were used as vehicle controls and oleic acid was used as fatty acid control. All treatments were incubated in quadruplicate.

Add back experiments were conducted as follows. HL-60 cells were incubated and treated with EPA as stated above. Cells were subsequently treated with 10 μM AA-861 and various concentrations (0.001, 0.01, 0.1 and 1.0 μM) of leukotriene B_4 (Cayman Chemical, Ann Arbor, MI) and various concentrations (0.001, 0.01, 0.1 and 1.0 μM) of 5(S)-HETE (Cayman Chemical, Ann Arbor, MI). Eicosapentaenoic acid treated HL-60 cells were subsequently treated with 5 μM baicalein and various concentrations (0.001, 0.01, 0.1 and 1.0 μM) of 12(S)-HETE (Cayman Chemical, Ann Arbor, MI).

Detection of apoptosis (flow cytometry). Detection of apoptosis by flow cytometry was performed as described previously using Annexin V-FITC and propidium iodide stains (BD Pharmingen, San Diego, CA) [8]. Flow cytometry was performed using a Becton-Dickenson FACScan flow cytometer and Cell Quest software version 1.2 (Becton-Dickenson, San Jose, CA).

Detection of apoptosis (TUNEL). Detection of apoptosis by TUNEL assay was performed using the *in situ* death detection, fluorescein kit (Roche, Mannheim, Germany). Approximately 20,000 cells were centrifuged at 500 rpm for 5 minutes (Cytospin3, Shandon, England). Cells were air dried and fixed with Carson solution. Slides were rinsed twice with PBS and were incubated in cytofix/cytoperm solution (100 μl) at 4°C for 20 minutes (Pharmagen, San Diego). Slides were rinsed in perm/wash buffer and allowed to dry. 50 μl of TUNEL reaction mixture (kit) was added to slides and incubated in a humidified chamber

for 60 minutes at 37°C. Hoechst stain (100 μl) was added and the slides were incubated for 30 minutes at 37°C. Slides were rinsed with PBS and protected with cover slips. Slides were analyzed with an Olympus BX51 fluorescent microscope (Olympus Optical Co., LTD., Japan) and images were captured with a cooled Q Color 3 camera (Q Imaging, Burnaby, BC Canada).

Detection of apoptosis (caspase-3 western blot). Cells were plated in sterile Costar six well cell culture plates (Corning, Corning, NY) at a concentration of 500,000 cells/ml. Cells were treated with 50 μM EPA in the presence of an enzyme inhibitor (10 μM ibuprofen, 10 μM NDGA, 5 μM bacalein, 10 μM AA-861, and 10 μM MK-886) for 12 hours. Cells were removed from plates and washed twice with PBS. Approximately 1 x 10^6 cells were lysed with 1X lysis buffer (10X = 30 mM $MgCl_2$, 100 mM NaCl, 5% (v/v) NP-40, and 100 mM Tris-HCl pH 8.0) plus DNase (Promega, Madison, WI), RNase (Ambion, Austin, TX), and protease inhibitor cocktail. Cell suspensions were incubated at 37°C for 10 minutes. After incubation 0.2 volumes of 4X loading buffer (Invitrogen, Carlsbad, CA) was added and samples were heated at 80°C for 2 minutes. Samples were loaded onto a 12% SDS-ploy acrylamide gel (Invitrogen, Carlsbad, CA) and run at 100 volts for 120 minutes in an Invitrogen Novex mini-cell (Invitrogen, Carlsbad, CA). Kaleidoscope prestained standard was used (Bio-Rad, Hercules, CA). After electrophoresis gels were transferred to a transfer cage. A “sandwich” was made with nitrocellulose and Whatmann 3mm papers (England). The transfer cage was then immersed in transfer buffer (800 ml methanol, ddH_2O to 4 liters, 9.7 g

Tris Base, and 57.6 g glycine) in a Hoeffer transfer apparatus (Scientific Instruments, San Francisco, CA). Transfer was run over night at 20 volts. After the protein has been transferred the nitrocellulose is stained with Ponceau S stain for 5 minutes. Stain was washed off with two washes of PBS for 10 minutes. Membranes were blocked for 1 hour with gentle agitation in blocking solution (5% (w/v) Carnation non-fat dry milk in PBS and 2% normal goat sera). After incubation, membranes were washed with TPBS (0.5% (v/v) Tween-20 (Fisher Scientific) and PBS) for 5 minutes. Membranes were incubated with caspase-3 (human) polyclonal antibody (Cayman Chemical, Ann Arbor, MI) diluted (1:750) in blocking solution overnight with gentle agitation at 4°C. Normal sera control membranes were also run using normal rabbit sera in lieu of the primary antibody. Primary antibody (and normal rabbit sera) was removed and membranes were washed twice with TPBS for 10 minutes with gentle agitation at room temperature. Membranes were incubated with anti-rabbit IgG (H+L) HRP conjugate (Promega, Madison, WI) diluted (1:2500) in blocking solution for 1 hour with gentle agitation at room temperature. After incubation membranes were washed twice for 10 minutes with TPBS. Membranes were rinsed once with PBS and subsequently developed using an ECL western blotting detection kit (Amersham Biosciences, Piscataway, NJ).

Statistics. Data are expressed as mean percent ± SE. Differences in mean percent were determined by one-way analysis of variance followed by REGWF

mean separation to determine statistical differences. Data were analyzed using CRUNCH version 4.0 statistical package (Crunch Software Corp., Oakland, CA).

Results:

Oleic acid, methanol and DMSO did not induce cell death (apoptosis and necrosis) and viability was maintained after 12-hour incubation [7;8]. Ibuprofen, NDGA, and baicalein did not induce apoptosis and necrosis and viability was maintained after a 12-hour incubation. AA-861 and MK-886 did not induce apoptosis and necrosis and did not affect viability at the lower concentrations at 12 hours. However, AA-861 (20 μM) and MK-886 (10 μM) significantly reduced viability as compared with media alone. Therefore, AA-861 (20 μM) and MK-886 (10 μM) were eliminated from the data set because they alone had effects on viability. These results indicate that the concentrations of enzyme inhibitors used alone had no significant effects on apoptosis, viability, and necrosis in HL-60 cells.

Polyunsaturated fatty acids can be metabolized by a variety of pathways that can be blocked with enzyme inhibitors (Figure 3.4.1.) The effects of various concentrations of ibuprofen, a cyclooxygenase 1 and 2 inhibitor, on viability, apoptosis, and necrosis in EPA treated HL-60 cells are shown in Figure 3.4.2. Treatment of HL-60 cells with 50 μM EPA (control) significantly reduced viability and significantly increased apoptosis and necrosis as compared with basal levels. Ibuprofen (5 μM) significantly reduced viability and increased necrosis as compared with control. Necrosis and viability were not significantly different

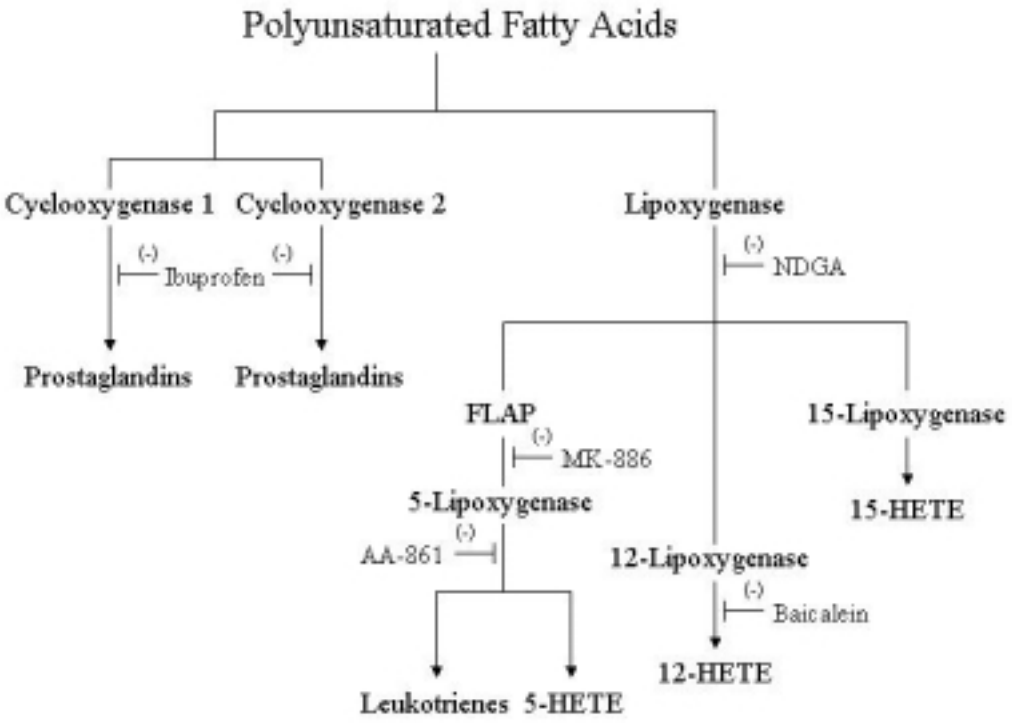

Figure 3.4.1. Schematic of the major pathways involved in the metabolism of polyunsaturated fatty acids and inhibitors of the various metabolic enzymes involved.

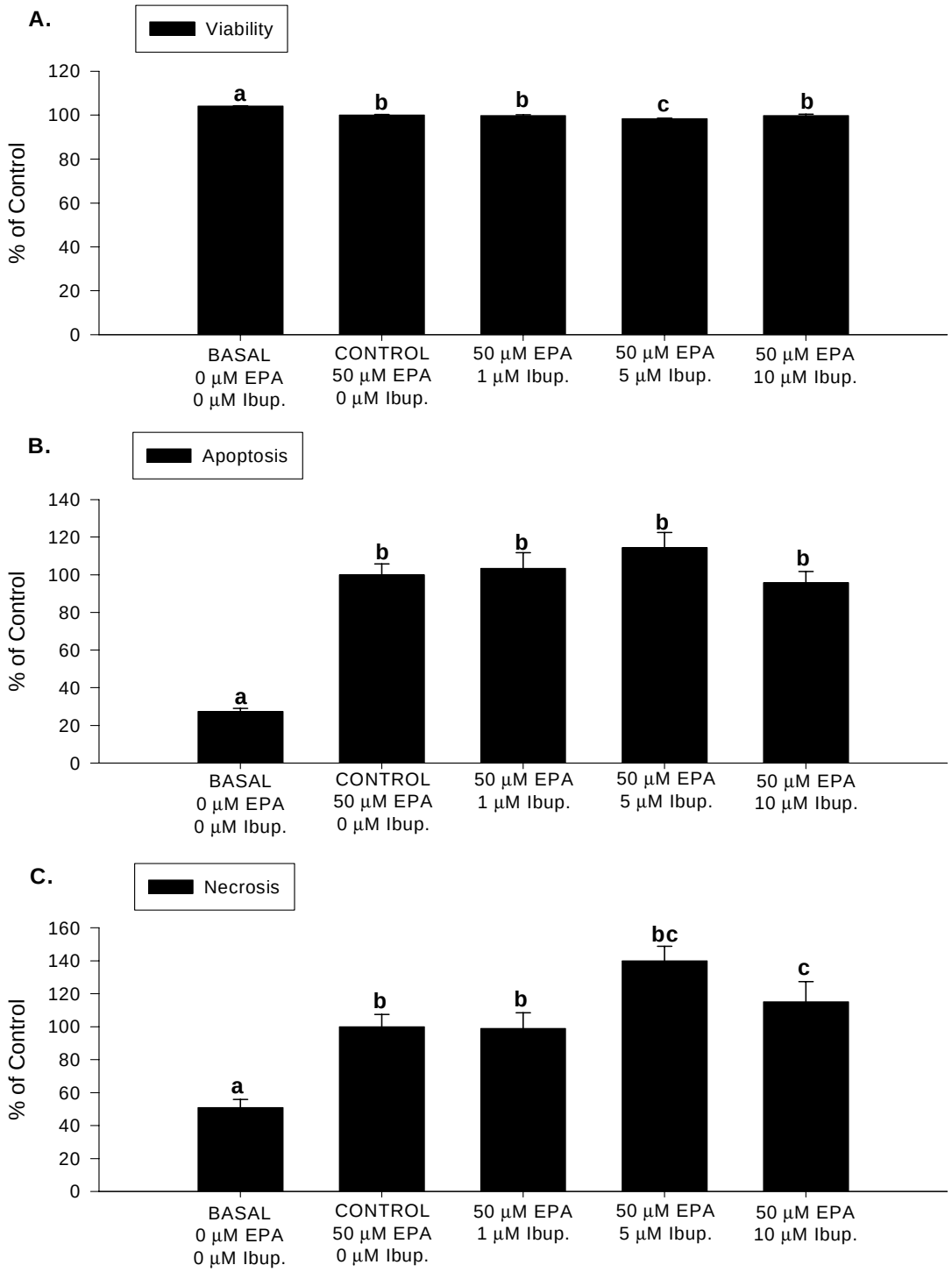

Figure 3.4.2. Effects of ibuprofen (COX 1 and 2 inhibitor) on viability, apoptosis, and necrosis of EPA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

between control and 1 μM ibuprofen. Apoptosis was not significantly different between control and 1, 5, and 10 μM ibuprofen after 12-hour incubation.

The effects of various concentrations of NDGA, a 5-, 12-, and 15-lipoxygeanse inhibitor, on viability, apoptosis and necrosis in EPA treated HL-60 cells are shown in Figure 3.4.3. Treatment of HL-60 cells with 50 μM EPA (control) significantly increased apoptosis as compared with basal levels. NDGA (10 μM) significantly reduced viability and necrosis as compared with control. Necrosis and viability were not significantly different between control and 1 and 5 μM NDGA. Apoptosis was not significantly different between control and 1, 5, and 10 μM NDGA after 12-hour incubation.

The effects of various concentrations of baicalein, a 12-lipoxygenase inhibitor, on viability and cell death in EPA treated HL-60 cells are shown in Figure 3.4.4. Treatment of HL-60 cells with 50 μM EPA (control) significantly reduced viability and significantly increased apoptosis as compared with basal levels. Baicalein (5 μM) significantly reduced viability as compared with control. Baicalein (1 and 10 μM) were not significantly different than control for viability. Apoptosis was significantly increased in HL-60 cells at baicalein concentrations of 1, 5, and 10 μM as compared with control. Necrosis was significantly different at 5 μM baicalein as compared with control after 12-hour incubation.

The effects of various concentrations of AA-861, a 5-lipoxygenase inhibitor, on viability and cell death in EPA treated HL-60 cells are shown in Figure 3.4.5. Treatment of HL-60 cells with 50 μM EPA (control) significantly

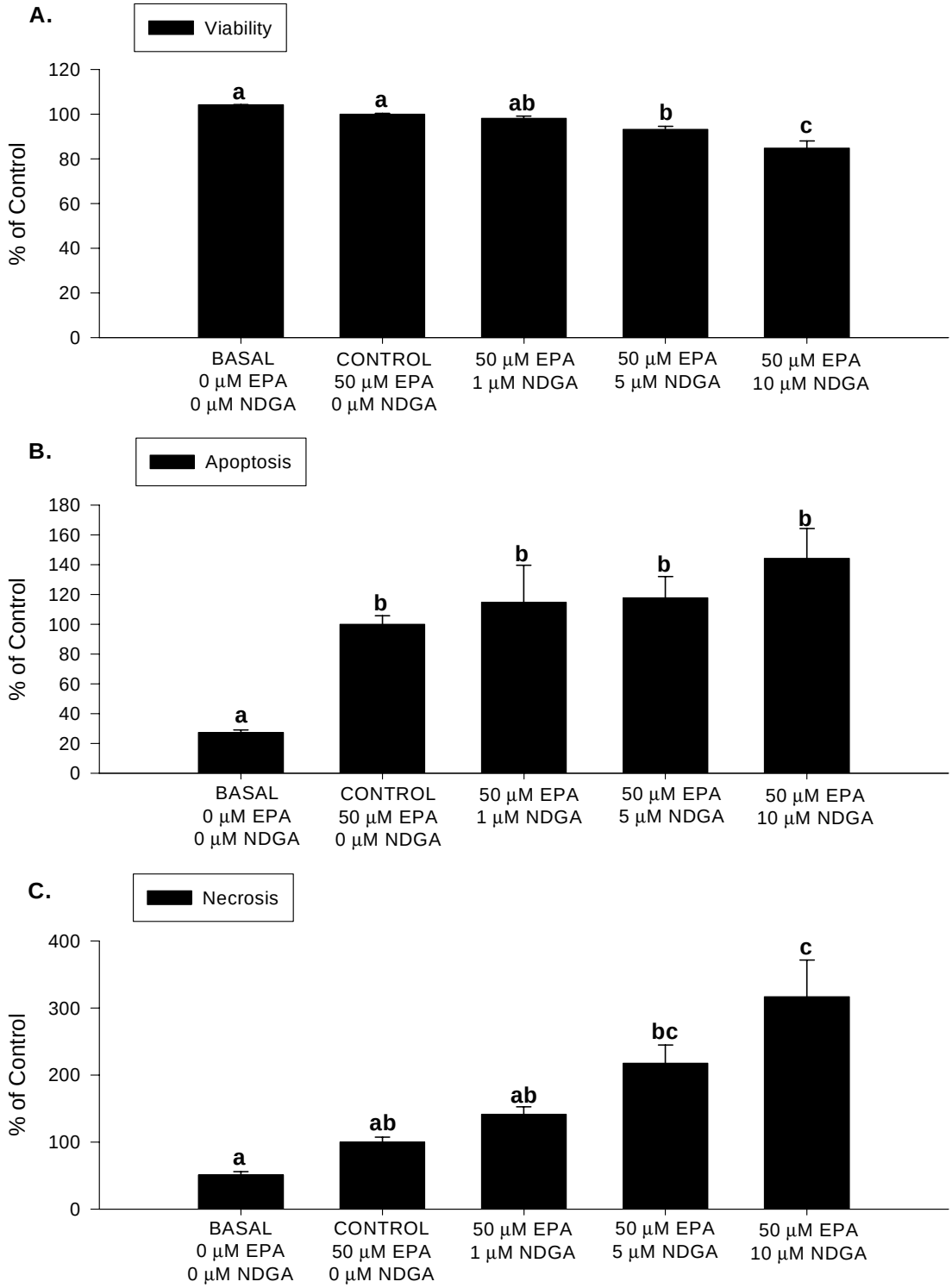

Figure 3.4.3. Effects of NDGA (5-, 12-, and 15-LO inhibitor) on viability, apoptosis, and necrosis of EPA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

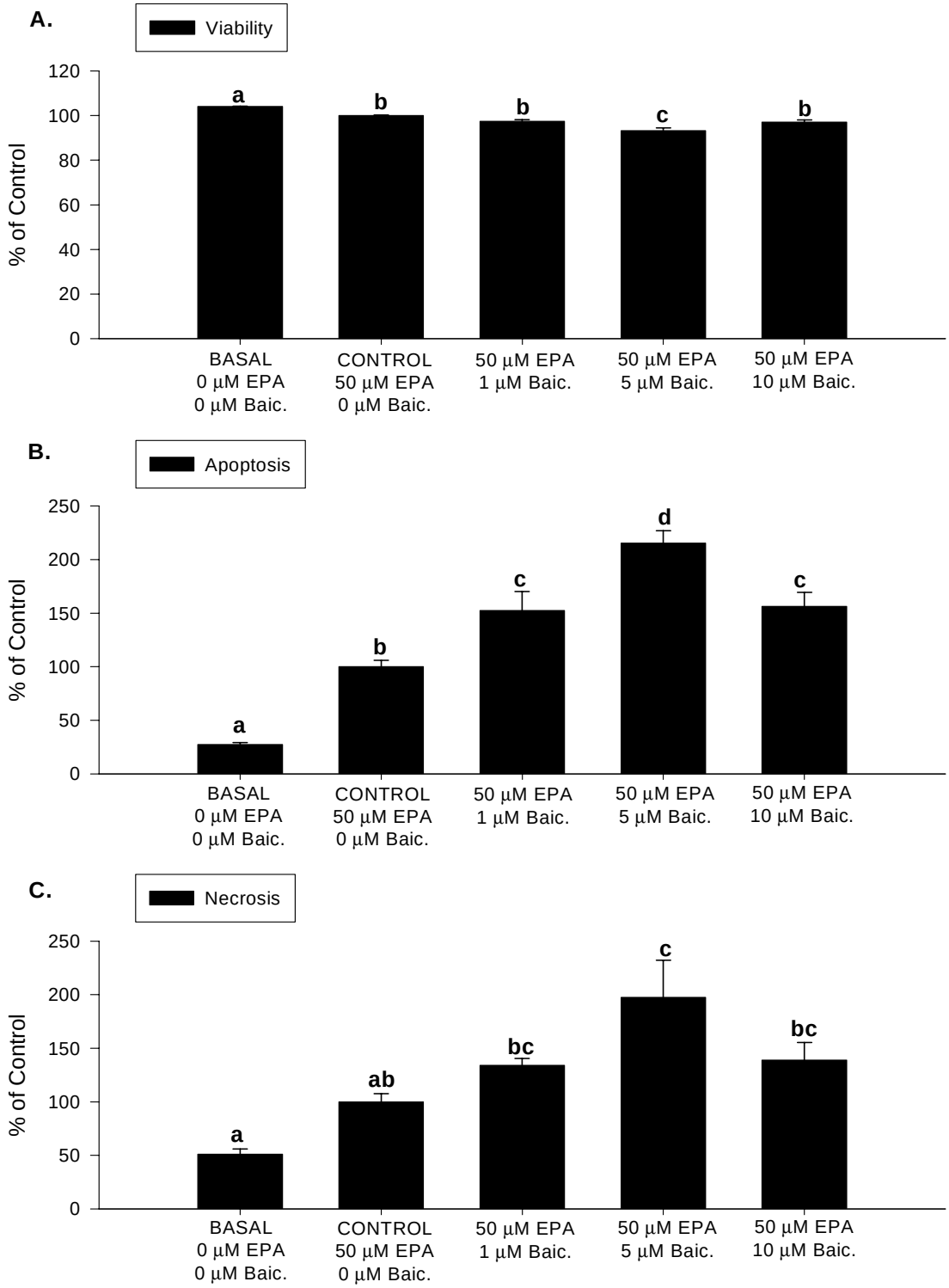

Figure 3.4.4. Effects of baicalein (12-LO inhibitor) on viability, apoptosis, and necrosis of EPA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c] Values sharing the same superscript letter are not different ($P < 0.05$).

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

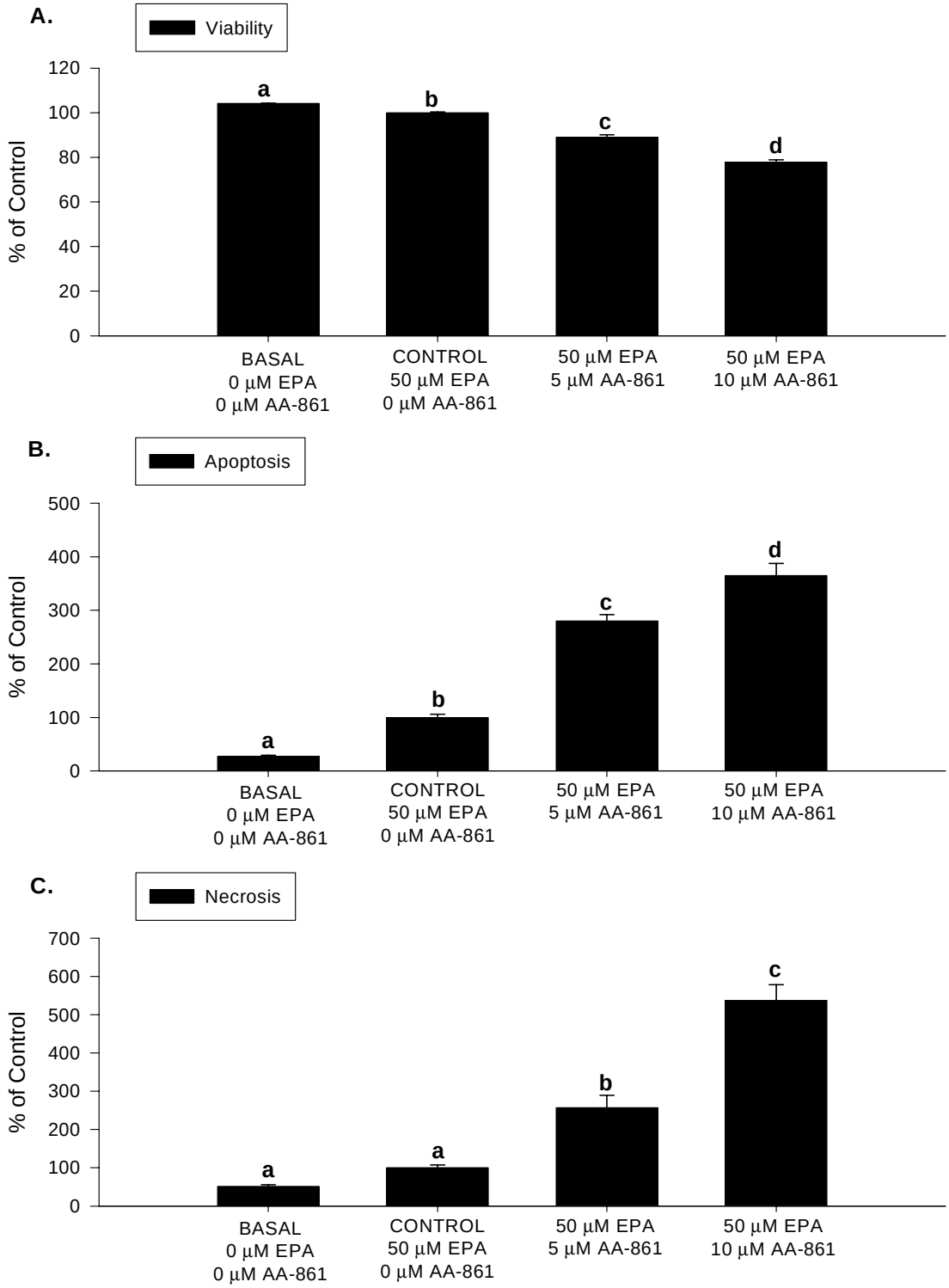

Figure 3.4.5. Effects of AA-861 (5-LO inhibitor) on viability, apoptosis, and necrosis of EPA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c,d] Values sharing the same superscript letter are not different ($P < 0.01$).

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

reduced viability and significantly increased apoptosis as compared with basal levels. AA-861 (5 and 10 μM) significantly reduced HL-60 cell viability as compared with control. AA-861 (5 and 10 μM) significantly increased cell death as compared with control after 12-hour incubation.

The effects of various concentrations of MK-886, a 5-lipoxygenase activating protein inhibitor, on viability and cell death in EPA treated HL-60 cells are shown in figure 3.4.6. Treatment of HL-60 cells with 50 μM EPA (control) significantly reduced viability and significantly increased apoptosis as compared with basal levels. MK-886 (1 and 5 μM) significantly reduced HL-60 cell viability as compared with control. MK-886 (1 and 5 μM) significantly increased apoptosis and necrosis as compared with control after 12-hour incubation.

The effects of various enzyme inhibitors tested on a TUNEL assay of EPA-treated HL-60 cells are shown in figure 3.4.7. EPA-treated HL-60 cells incubated with ibuprofen, NDGA, baicalein, and AA-861 (Fig. 3.4.7*C-F*, respectively) all exhibited a positive TUNEL assay, indicating the presence of apoptotic cells.

The effects of various enzyme inhibitors on whole cell extracts western blot analysis of celaved-caspase-3 of EPA-treated HL-60 cells are shown in figure 3.4.8. Cleaved caspase-3 is evident in all lanes after 12-hour incubation. Densities as a percent of negative control are shown below each lane. All treatment groups except for AA-861 had an increase in the density of cleaved caspase-3. At 12-hours the amount of intact cleaved caspase-3 is less than the negative control due to continued cleavage of proteins by activated proteases for

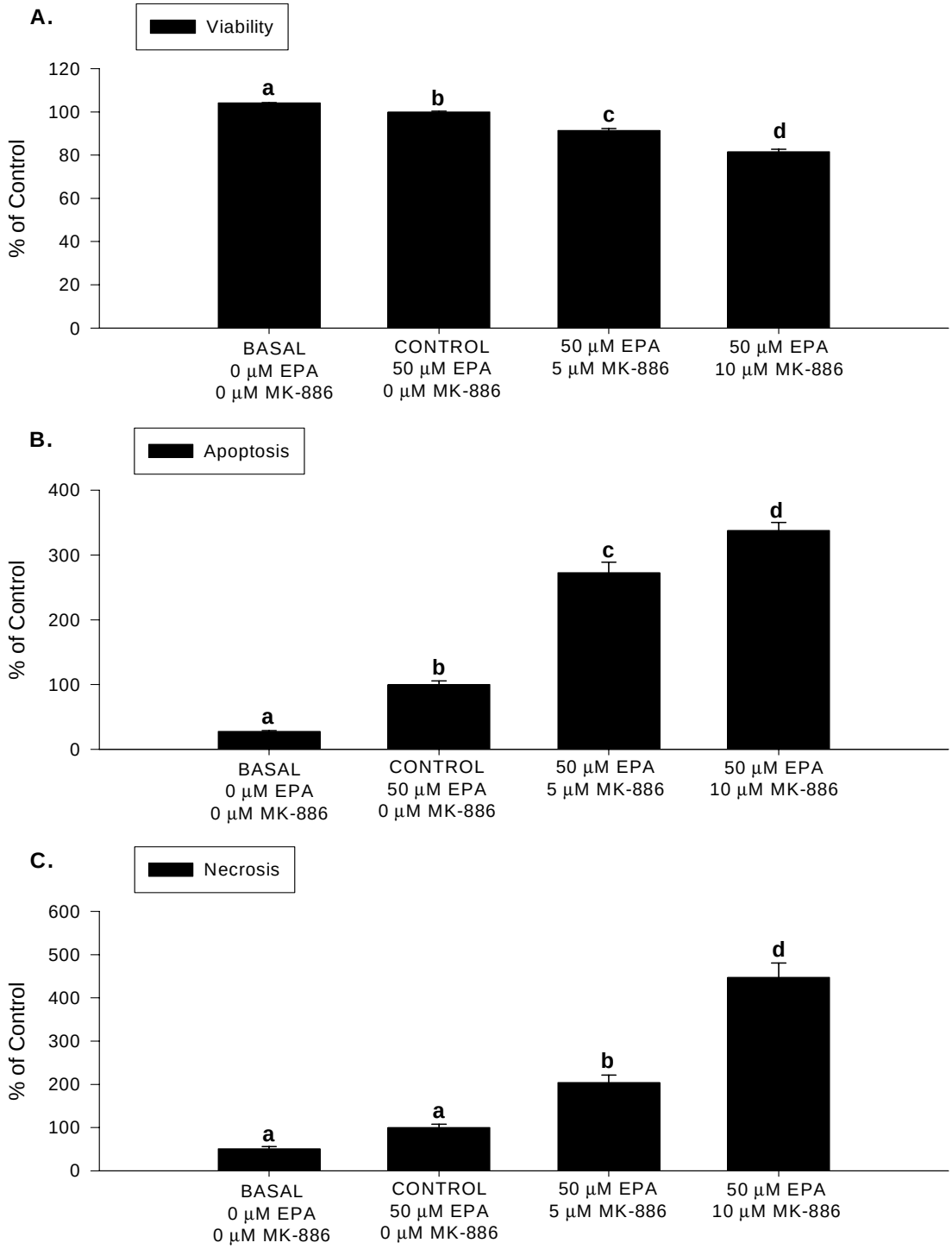

Figure 3.4.6. Effects of MK-886 (FLAP inhibitor) on viability, apoptosis, and necrosis of EPA-treated HL-60 cells after 12-hour incubation.[1]

[a,b,c,d] Values sharing the same superscript letter are not different ($P < 0.01$).

[1] Mean ± SE of the % of control for viability, apoptosis and necrosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

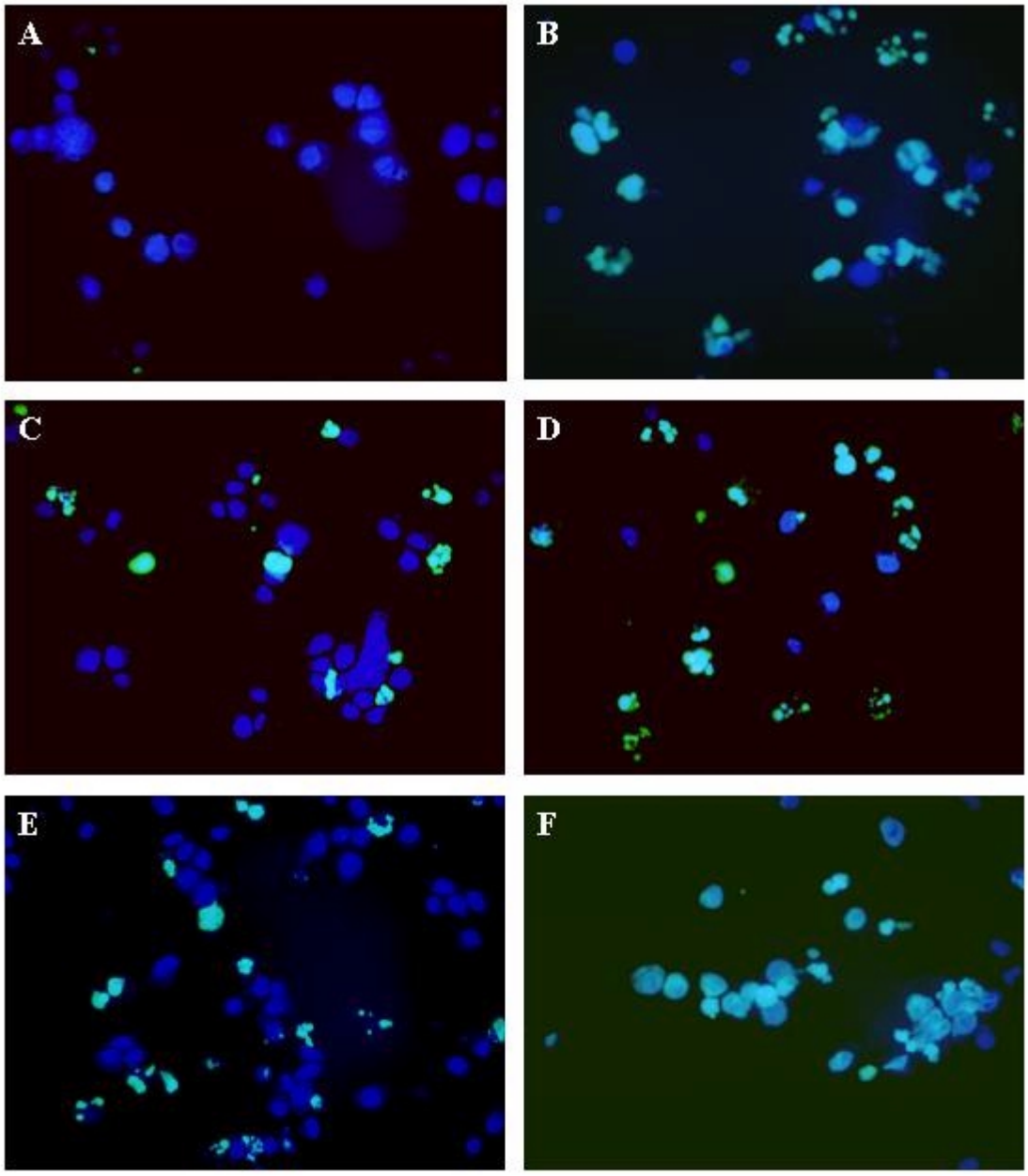

Figure 3.4.7. The effects of various enzymes inhibitors on TUNEL assay of EPA-treated HL-60 cells after 12-hour incubation.[1]

A. Negative Control; B. Positive Control (5 μM CAM); C. 50 μM EPA plus 10 μM ibuprofen; D. 50 μM EPA plus 10 μM NDGA; E. 50 μM EPA plus 5 μM baicalein; F. 50 μM EPA plus 10 μM AA-861

[1]Positive TUNEL indicated by green fluorescence; All photos 400x

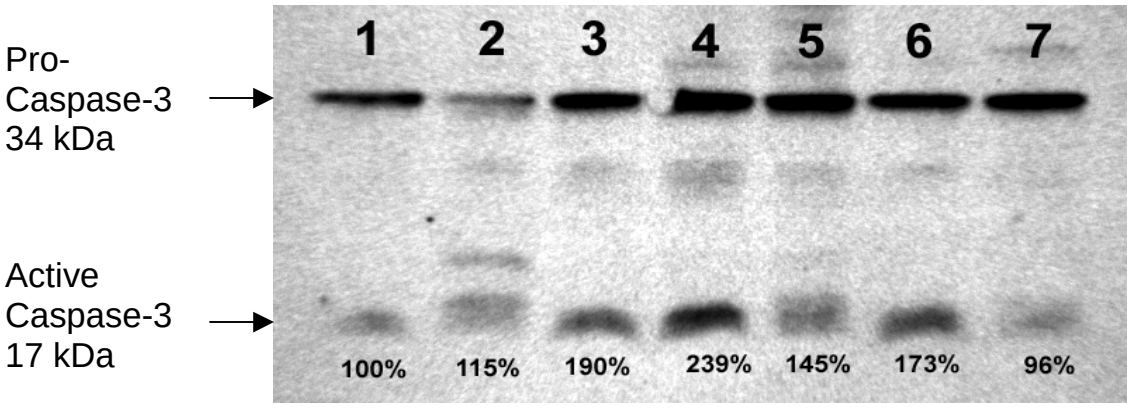

Figure 3.4.8. The effects of various enzymes inhibitors on active caspase-3 western blot of EPA-treated HL-60 cells after 12-hour incubation.[1]

[1] % of negative control listed below each lane for active caspase-3.

Lanes: 1 = Negative Control, 2 = Positive Control (5μM CAM), 3 = 50 μM EPA, 4 = 50 μM EPA plus 10 μM Ibuprofen, 5 = 50 μM EPA plus 10 μM NDGA, 6 = 50 μM EPA plus 5 μM Baicalein, 7 = 50 μM EPA plus 10 μM AA-861.

Gel: 12 % SDS-ployacrylamide gel

Running Conditions: 120 minutes at 100 volts.

AA-861 treated HL-60 cells.

The effects on apoptosis of various concentrations of 12-hydroxyeicosatetraenoic acid (12-HETE) on 5 μM baicalein-EPA treated HL-60 cells are shown in figure 3.4.9. Apoptosis was not significantly different between control and 1.0, 0.1, 0.01, and 0.001 μM 12-HETE after 12-hour incubation.

The effects on apoptosis of various concentrations of 5-hydroxyeicosatetraenoic acid (5-HETE) on 10 μM AA-861-EPA treated HL-60 cells are shown in figure 3.4.10. Apoptosis was not significantly different between control and 1.0, 0.1, 0.01, and 0.001 μM 5-HETE after 12-hour incubation.

The effects on apoptosis of various concentrations of LTB_4 on 10 μM AA-861-EPA treated HL-60 cells are shown in figure 3.4.11. Apoptosis was not significantly different between control and 1.0 μM LTB_4 after 12-hour incubation. LTB_4 (0.1, 0.01, and 0.001 μM) significantly reduced apoptosis as compared with control.

Discussion:

We have previously shown that treatment of HL-60 cells with eicosapentaenoic acid (EPA; 20:5 n-3) increased apoptosis and secondary necrosis and reduced viability in a concentration and time-dependent manner. However, it was not known if the induction of cell death by EPA involved the eicosanoid products of 5- or 12-lipoxygenase (LO) and/or cyclooxygenase 1 and 2 (COX). This study demonstrates that the increase in apoptosis and secondary

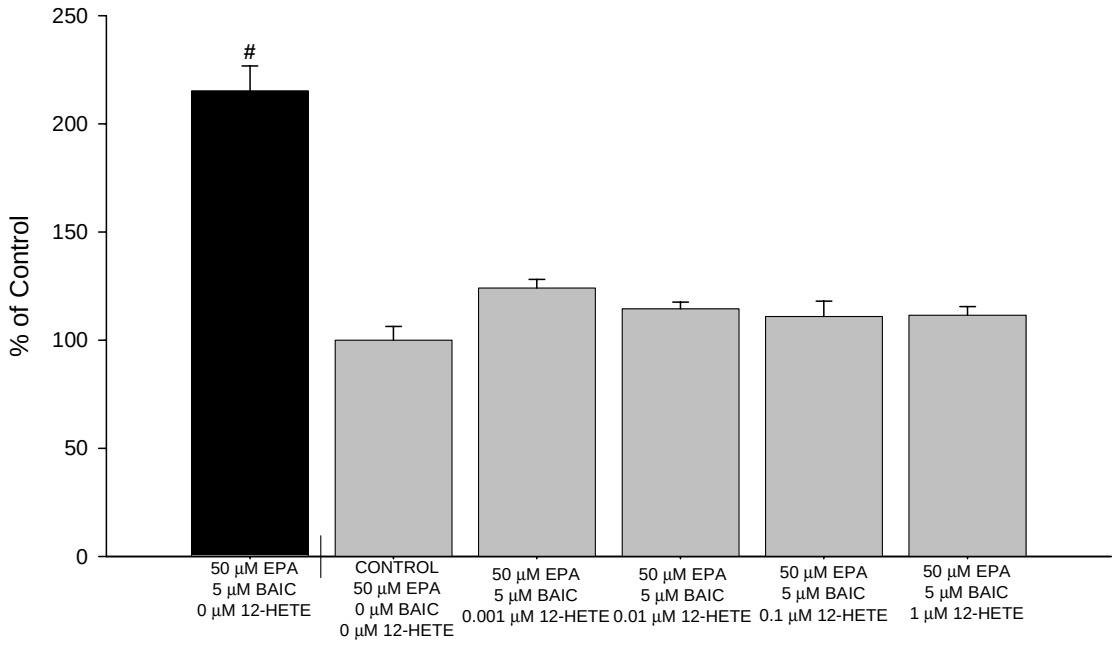

Figure 3.4.9. The effects of various concentrations of 12-HETE on apoptosis of baicalein and EPA-treated HL-60 cells after 12 hour incubation.[1,2]

[1] Mean ± SE of the % of control for apoptosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM EPA and 5 μM baicalein.

[#] Data from Figure 3.4.4.

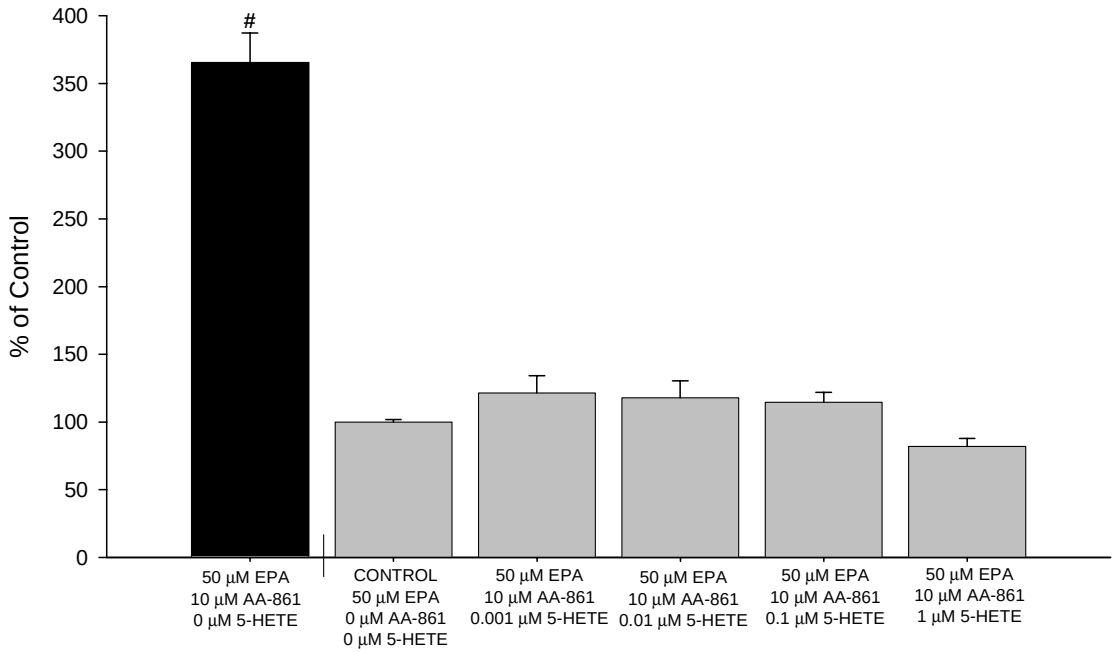

Figure 3.4.10. The effects of various concentrations of 5-HETE on apoptosis of AA-861 and EPA-treated HL-60 cells after 12 hour incubation.[1,2]

[1] Mean ± SE of the % of control for apoptosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM EPA and 10 μM AA-861.

[#] Data from Figure 3.4.5.

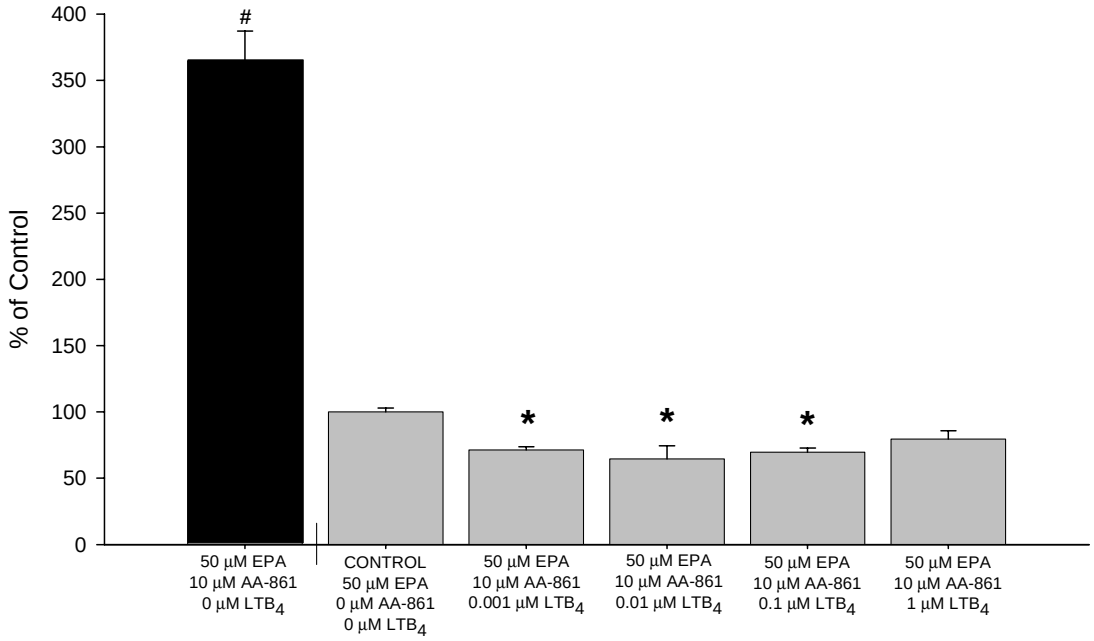

Figure 3.4.11. The effects of various concentrations of LTB_4 on apoptosis of AA-861 and EPA-treated HL-60 cells after 12 hour incubation.[1,2]

* For a given variable, value different from control (P<0.05)

[1] Mean ± SE of the % of control for apoptosis as measured using flow cytometry with propidium iodide and annexin V-FITC staining.

[2] HL-60 cells were pretreated with 50 μM EPA and 10 μM AA-861.

[#] Data from Figure 3.4.5.

necrosis due to EPA was increased further with inhibitors of either 5- or 12-lipoxygenase in HL-60 cells. Furthermore, eicosanoid add-back experiments showed that inhibition of the downstream metabolic processing of EPA to 5-HETE and LTB_4 (by inhibition of 5-LO) and/or to a lesser extent to 12-HETE (by inhibition of 12-LO) increased apoptosis and secondary necrosis in HL-60 cells. The inhibition of COX 1 and 2 did not affect apoptosis in EPA treated HL-60 cells.

LTB_4 is a natural inflammatory ligand, potent chemoattractant of neutrophils, and stimulator of leukocytes adhesion to endothelial cells [22;23]. Inhibition of 5-lipoxygenase with AA-861 in EPA-treated HL-60 cells significantly increased apoptosis and secondary necrosis as well as significantly decreased viability. Inhibition of 5-lipoxygenase activating protein (FLAP) with MK-886 produced similar cellular responses to inhibition of 5-LO with AA-861. FLAP is a membrane associated protein that is critical in fatty acid metabolism by 5-LO [24]. Furthermore, adding back 5-HETE to EPA-treated HL-60 cells incubated with AA-861 returned the rate of apoptosis to a level that was not different from EPA-treated HL-60 cells alone (Figure 3.4.10). All concentrations of 5-HETE tested returned apoptosis to control levels, implying that 5-HETE is an important compound in the regulation of apoptosis in EPA-treated HL-60 cells. Adding back LTB_4 to EPA-treated HL-60 cells incubated with AA-861 had the greatest affect on apoptosis (Figure 3.4.11). At the lower concentrations of LTB_4 tested (0.1-0.001 μM) apoptosis was significantly lower than control. This implies that LTB_4 is critical to the regulation of apoptosis in EPA-treated HL-60 cells and that in its absence cell survival will be severely reduced. This data concurs with

human neutrophil data in which inhibition of 5-LO or FLAP decreased cell survival in lipopolysaccharide-treated neutrophils [18]. Thus, these results indicate that 5-LO and its products, particularly LTB_4, are critical in the regulation of apoptosis in EPA-treated HL-60 cells.

Inhibition of 12-LO with baicalein in EPA-treated HL-60 cells reduced cell viability and increased apoptosis and necrosis, with a significant increase in apoptosis across all concentrations tested. When 12-HETE was added back to EPA-treated HL-60 cells incubated with 5 μM baicalein, apoptosis is returned to a level that is not different from EPA-treated HL-60 cells alone (Figure 3.4.9). Baicalein (5 μM) had the greatest affect on increasing apoptosis in EPA-treated HL-60 cells (Figure 3.4.4). However, at the highest concentration tested (10 μM) did not differ from control for viability. It is possible that the highest concentration of baicalein was nonspecifically binding to other lipoxygenases and possibly other enzymes. Add back of 12-HETE, at all concentrations, returned apoptosis to control levels. This implies that 12-HETE is an important compound in the regulation of apoptosis in EPA-treated HL-60 cells.

The lipoxygenase pathway consists of three primary pathways: 5-LO, 12-LO, and 15-LO (Figure 3.4.1). 5-lipoxygenase metabolizes AA to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is quickly reduced to 5-HETE or LTA_4. LTA_4 can subsequently be converted to the cysteinyl leukotrienes: LTC_4, LTD_4, and LTE_4 [25]. LTA_4 can also be converted to LTB_4. The cysteinyl leukotrienes are known as slow-reacting substance of anaphylaxis and are intimately involved in asthma and other hypersensitivity reactions [25].

Cysteinyl leukotrienes increase permeability in post capillary venules, stimulate mucus secretions, and potentiate bronchial constriction [26]. Leukotrienes derived from EPA are far less pro-inflammatory than are leukotrienes derived from AA. Furthermore, EPA derived leukotrienes compete for binding sites with AA-derived leukotrienes [22]. 12-LO and 15-LO metabolize AA to form 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE), respectively, and EPA to 12-hydroxyeicosapentaenoic acid (12-HEPE) and 15-hydroxyeicosapentaenoic acid (15-HEPE), respectively. 12-LO has been shown to be present in the skin of rats and when stimulated with bradykinin and platelet activating factor, both potent pro-inflammatory compounds, produced 12-HETE and hepoxilins [27]. Furthermore, 12-HETE administered intradermally increased vascular permeability and acute inflammation [27]. 15-HETE can be metabolized to form the lipoxins (lipoxins A_4 and B_4), which are considered important compounds in inflammation and immunity [26]. Lipoxins decrease adhesion and transmigration of neutrophils and inhibit chemotaxis induced by LTB_4 and fMLP [28]. Lipoxins A_5 and B_5, derived from EPA, are as biologically potent as AA-derived lipoxins A_4 and B_4 [29].

Flow cytometry after dual-staining with annexin V-FITC in combination with propidium iodide (PI) was used to differentiate between viable, apoptotic and secondary necrotic HL-60 cells. Viable cells do not stain with either dye, apoptotic cells with only annexin V-FITC, and secondary necrotic cells with both annexin V-FITC and PI. *In vitro,* secondary necrotic cells have been defined to be in the phase following apoptosis [30;31]. Annexin V-FITC binds to

externalized phosphatidylserine, which prior to apoptosis is distributed on the inner leaflet of the cell membrane. *In vivo*, externalized phosphatidylserine is a physiological marker that macrophages use to recognize and engulf apoptotic cells before they degrade via secondary necrosis [32]. Our system consists of a homogenous cell culture that contains no phagocytic cells. Therefore, apoptotic cells will degrade via secondary necrosis. In this study, HL-60 cells with both stains were taken to be due to apoptosis leading to secondary necrosis. We confirmed, in previous studies, the presence of apoptotic HL-60 cells by the use of DNA fragmentation and TUNEL assays [7;8]. Apoptosis remained relatively constant between 8 and 24-hours of incubation indicating that cells were undergoing apoptosis at a relatively constant rate and that the increase in secondary necrosis was due to apoptotic cells continuing to degrade via secondary necrosis [7].

Determination of apoptosis by terminal dUTP nick end-labeling (TUNEL) assay is a technique that fluorescently visualizes apoptotic DNA fragmentation [33;34]. TUNEL assay was positive in EPA-treated HL-60 cells for all four enzyme inhibitors tested: ibuprofen, NDGA, baicalein, and AA-861. Although treatment with ibuprofen exhibited a positive TUNEL assay, there was no change in apoptosis in EPA-treated HL-60 cells as measured by flow cytometry. This was most likely due to an increase in apoptosis caused by EPA and not ibuprofen since no measurable increase in apoptosis was detected by analysis with flow cytometry. We previously demonstrated that EPA alone will induce apoptosis in HL-60 cells at 12-hours [7]. Furthermore, the number of apoptotic

cells in EPA-treated HL-60 cells incubated with ibuprofen is less than the number of apoptotic cells visible in EPA-treated HL-60 cells incubated with NDGA, baicalein, and AA-861 (Figure 3.4.7 *C-F*, respectively).

This study demonstrated the pro-caspase-3 is cleaved to active caspase-3 with the treatment of EPA and various enzyme inhibitors. As stated before, all treatment groups except for AA-861 had an increase in the density of cleaved caspase-3. The decrease in cleaved caspase-3 is most likely due to the fact that AA-861 induces significant amounts of apoptosis in EPA-treated HL-60 cells. At this stage of apoptosis, the degradation of proteins, including caspase-3, is great throughout the cell. Therefore, at 12-hours the amount of intact cleaved caspase-3 is less than the negative control due to continued cleavage of proteins by activated proteases for AA-861 treated HL-60 cells. Ibuprofen exhibited a positive cleaved caspase-3 western blot. This is most likely due to the induction of apoptosis by EPA rather than the inhibition of COX 1 and 2 by ibuprofen. Furthermore, ibuprofen exhibited the most intense signal of all inhibitors tested due to the fact that 12-hour incubation is an excellent time point for EPA-treated HL-60 cells and much of the protein was still intact. Caspase stands for cysteine aspartate-specific proteases [35] and is considered the workhorse of apoptosis. Activation of caspases works through a caspase cascade starting with the initiator caspases: caspase-8 and caspase-9 [36]. Caspase-8 cleaves and activates caspase-3, -6, and -7 [36]. The most prevalent caspase in the cell is caspase-3 [36]. Procaspase-3 (inactive caspase) exists as a 34 kDa protein. Once procaspase-3 is cleaved it becomes active and yields a heterodimer

consisting of p11 and p20 subunits [35]. Activated caspase-3 will cleave many proteins such as cytoskeletal proteins and proteins involved in DNA repair such as polyADP-ribose polymerase (PARP) [35]. Caspase-3, along with caspases-6 and -7 are referred to as the “executioner caspases” [36].

We conclude that the LO and not the COX pathways are involved in the regulation of apoptosis in EPA-treated HL-60 cells. Several methods of apoptosis detection were used including flow cytometry, TUNEL assay, and activated caspase-3 western blots to substantiate the results and to show that inhibition of LO, particularly 5-LO, increased apoptosis in EPA-treated HL-60 cells. Furthermore, this study clearly demonstrated that the products of the LO enzymes, particularly LTB_4, are critical in the regulation of apoptosis in EPA-treated HL-60 cells. This study provides a further understanding of the effects of EPA on HL-60 cell apoptosis, at the cellular and molecular level, and suggests that dietary intake of n-3 fatty acids may increase apoptosis in inflammatory cells, thereby limiting neutrophil mediated tissue injury and enhancing the resolution of acute inflammation such as that seen in acute lung injury and ARDS.

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V. Apoptotic Gene Expression in Eicosapentaenoic Acid and γ-Linolenic Acid-Treated HL-60 Cells

Abstract:

Polyunsaturated fatty acids from the n-3 and n-6 series have been shown to induce apoptosis in a variety of cell types as well as be beneficial in a variety of health problems, such as arthritis and acute respiratory distress syndrome (ARDS). We have previously shown, using HL-60 cells as a model for the human neutrophil, that eicosapentaenoic acid (EPA, 20:5 n-3) and the combination of EPA and γ-linolenic acid (GLA, 18:3 n-6) induce apoptosis in a concentration- and time-dependent manner. However, we do not know the gene profile of the EPA/GLA-induced apoptotic HL-60 cells. Thus, the objective of this study was to determine the apoptotic gene profile of HL-60 cells treated with the combination of EPA and GLA. HL-60 cells were incubated with 50 μM EPA and 20 μM GLA for 12 hours. Apoptotic gene profiles were assessed using GEArray original series human apoptosis-2 gene arrays. Total RNA was extracted and probes were synthesized with a primer mix unique for each array. Arrays were hybridized and developed in a non-isotopic manner. Arrays were visualized using a 16-bit CCD camera. The apoptotic gene profile for no treatment and EPA/GLA-treated HL-60 cells after 12-hours differed from each other. When comparing density values as a percent of control, all genes assayed were down-regulated. However, comparison of the relative intensities of several genes in the arrays differed between control and treatment groups. For example, Bad,

Bax, and Fas were down regulated and Bax, Bcl-2, and p53 were up regulated as compared with control. However, due to the unreliable data set no definitive conclusions can be made. The ability to elucidate the apoptotic gene profile of EPA/GLA-treated HL-60 cells may lead to new therapies in the induction of neutrophil apoptosis to enhance the resolution of inflammation and reduce tissue injury in patients with ARDS.

Key Words: Fish Oil, ARDS, Inflammation, Gene Profile, Neutrophil, Polyunsaturated Fatty Acids

Introduction:

Polyunsaturated fatty acids (PUFA) from the n-3 and n-6 series have been shown to induce apoptosis in a variety of cell types [1-5]. Eicosapentaenoic acid (EPA; 20:5 n-3), found in fish oil, and γ-linolenic acid (GLA; 18:3 n-6), found in borage, evening prime rose, and blackcurrant seed oils, have beneficial effects in a variety of health problems such as inflammation, cardiovascular disease, sepsis, arthritis, and acute respiratory distress syndrome (ARDS) [1;6-8]. The beneficial aspect of EPA and GLA may be through the induction of cellular apoptosis of the offending cell, i.e., neutrophils in ARDS.

Our laboratory has previously shown that enteral nutrition supplemented with EPA and GLA, reduced the number of neutrophils in the bronchoalveolar lavage fluid from patients and animals with ARDS [8;9]. We hypothesized that this reduction in neutrophils may be a direct result of EPA- and GLA-induced

neutrophil apoptosis. We subsequently demonstrated, using the human promyelocytic leukemia cell line HL-60 as model for the human neutrophil, that EPA and the combination of EPA and GLA induce apoptosis in a time- and concentration-dependent manner *in vitro* [3]. Furthermore, we demonstrated that lipoxygenase enzymes and their products are involved in the regulation of HL-60 cell apoptosis [10-12]. However, we do not know the gene profile of PUFA-induced apoptotic HL-60 cells. Recent studies of gene expression in undifferentiated HL-60 cells has revealed the expression of mRNA for the apoptotic genes bak, bik, bax, bad, bcl-2, bcl-x, bcl-w, bfl-1, fas, and caspases 1-4 and 7-10 [13]. After HL-60 cells were stimulated to differentiate, bak, blc-w, bfl-1, fas, and caspase 1 and 9 were up-regulated, whereas bik, bcl-2, and caspase 2, 3, and 10 were down-regulated [13]. We hypothesize that treatment of HL-60 cells with the combination of EPA and GLA will increase the expression of pro-apoptotic genes and will decrease the expression of anti-apoptotic genes. Thus, the objective of this study is to determine the apoptotic gene profile of HL-60 cells treated with the combination of EPA and GLA.

Materials and Methods:

Cells: The human promyelocytic leukemia cell line HL-60 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium with phenol red supplemented with HEPES (25 mM; BioWhittacker, Walkersville, MD), 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO), and 1% L-

glutamine (BioWhittaker, Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % room air in a NAPCO model 5410 incubator (Precession Scientific, Chicago, IL).

Treatments: All chemicals and reagents were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Cell number and viability were assessed using a hemacytometer and trypan blue dye exclusion technique. Cells were plated in sterile Costar 6 well cell culture plates (Corning, Corning, NY) at a concentration of 1 x 10^7 cells/ml. Cells were treated with 50 μM EPA and 20 μM GLA. Cells were treated for 12 hours only.

Detection of apoptotic genes: Treated cell cultures were subjected to total RNA extraction using the RNAqueous™ Kit (Ambion, Austin, TX). Cells were pelleted and washed then were subjected to lysis using the reagents and protocol provided in the RNAqueous™ Kit. Samples were centrifuged through RNA binding filters (kit) at 11,000 x *g* for 1 minute. RNA was eluted from the filter using 25 μL of 2X nuclease-free dH_2O heated to 70°C, followed by a brief centrifugation. Lithium chloride precipitation was performed to remove carbohydrates and gross DNA contamination. Briefly, RNA was mixed with 0.5 volume LiCl precipitation solution (kit) and incubated at –20°C for 30 minutes. The solution was microcentrifuged at top speed for 15 minutes. Then pellet was washed with ice cold 70% ethanol and recentrifuged. The pellet was raised in 25 μl TE buffer. Five microliters of total RNA was diluted in 295 μL of TE buffer in a

quartz microcuvette and analyzed photometrically at wavelengths of 260 nm and 280 nm in a UV-160A dual beam spectrophometer (Shimadzu Corp. Kyoto, Japan). Samples were assessed for quantity and purity. Samples falling outside the A_{260}/A_{280} ratio of 1.8-2.1 were considered to be either DNA or protein contaminated and were re-exposed to the RNA binding and washing protocol.

Probe synthesis: Probes were synthesized using GEArray ampolabeling-LPR kit (SuperArray Bioscience Corp., Frederick, MD). An annealing mixture was prepared using 10 μg of total RNA, 2 μl of buffer P (kit) and adjusted to a total volume of 20 μl with Rnase-free H_2O. Samples were heated briefly to 70°C for 3 minutes in a GeneAmp 2700 PCR system (Applied Biosystems, Foster City, CA) then cooled to 37°C for 10 minutes. RT cocktail (20 μL), which included 16 μL of 5X Nonrad-GEAlabeling buffer (Buffer BN, kit), 16 μL of Rnase-free water, 4 μL RNAse Inhibitor (kit), and 4 μL reverse transcriptase (kit), was added to the 20 μl of annealing mixture. This was then incubated at 37°C for 25 minutes then heated to 85°C for 5 minutes to hydrolyze the RNA and to inactivate the reverse transcriptase. LPR cocktail (60 μl), which included 72 μl buffer L (kit), 36 μl buffer AF (array kit), which is a primer mix unique for each array type, 8 μl biotin-16-dUTP (Boehringer Mannheim, Germany), and 4 μl DNA polymerase (kit), was added to the 40 μl of the RT reaction mix. This was run on a thermal cycler as follows: 85°C for 5 minutes, 30 cycles of 85°C for 1 minutes, 50°C for 1 minute, 72°C for 1 minutes, then 72°C for 5 minutes. The LPR reaction was immediately

stopped by adding 10 μl of buffer C (kit) to the reaction and chilling on ice. cDNA probes were denatured by heating the LPR reaction mix to 94°C for 2 minutes.

Hybridization: GEArray original series human apoptosis-2 gene arrays (SupperArray) were prehybridized in hybridization tubes (Fisher Scientific, Pittsburgh, PA) with 10 mL of GEAprehyb hybridization solution spiked with sheared, heat-denatured salmon sperm DNA (Gibco-BRL, Gaithesburg, MD) to a final concentration of 100 μg/ml at 68°C in an Isotemp® Hybridization Incubator (Fisher Scientific) for 2 hours at 5 rpm. The entire volume of denatured probe was added to 5 ml of warmed GEAprehyb. Following prehybridization, GEAprehyb was discarded and 5 mL of GEAhyb hybridization buffer was added to the membrane and hybridized overnight at 68°C and 5 rpm. Membranes were washed, prepped, blocked, and developed according to the GEArray™ protocol for non-isotopic detection.

Chemiluminescent Detection: Chemiluminescent detection of gene expression was assessed on a FluroChem 8000 imager (Alpha Innotech, San Leandro, CA). Expression of specific genes was measured by densitometry and normalized with internal membrane hybridization controls by expressing the intensity of experimental genes as a percentage intensity/density of the controls. Experimental and control treatments were normalized and contrasted to assess differential gene expression.

Results:

GEArray original series human apoptosis-2 gene arrays of 50 μM EPA and 20 μM GLA treated and no treatment (control) HL-60 cells after 12-hour incubation are shown in Figure 3.5.1. There is the clear presence of certain genes impregnated on each of the arrays. The brightest spots in positions 5G, 6G, 7G, 8E, 8F, and 8G are internal controls of the Glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene. Beta actin (3G and 4G) is also an internal control. pUC 18 (1G and 2G) is the negative control.

The apoptotic gene profile of EPA and GLA treated HL-60 cells after 12-hour incubation is presented in Table 3.5.1. Table 3.5.1 gives a summary of the presence and intensity of each gene impregnated on the membrane.

The apoptotic gene profile control HL-60 cells (no treatment) after 12-hour incubation is presented in Table 3.5.2. Table 3.5.2 gives a summery of the presence and intensity of each gene impregnated on the membrane.

Comparison of the relative apoptotic gene intensities of control and the combination of EPA and GLA-treated HL-60 cells after 12-hour incubation are shown in figure 3.5.3.

Percent of control for each gene in EPA and GLA treated HL-60 cells is presented in Table 3.5.4. Genes are presented as an integrated density value (IDV), which is the sum of all pixel values after background correction:

$$IDV = \Sigma \text{ (each pixel value-background)}$$

Overall, all genes appear to have been down-regulated when comparing control with treated gene arrays.

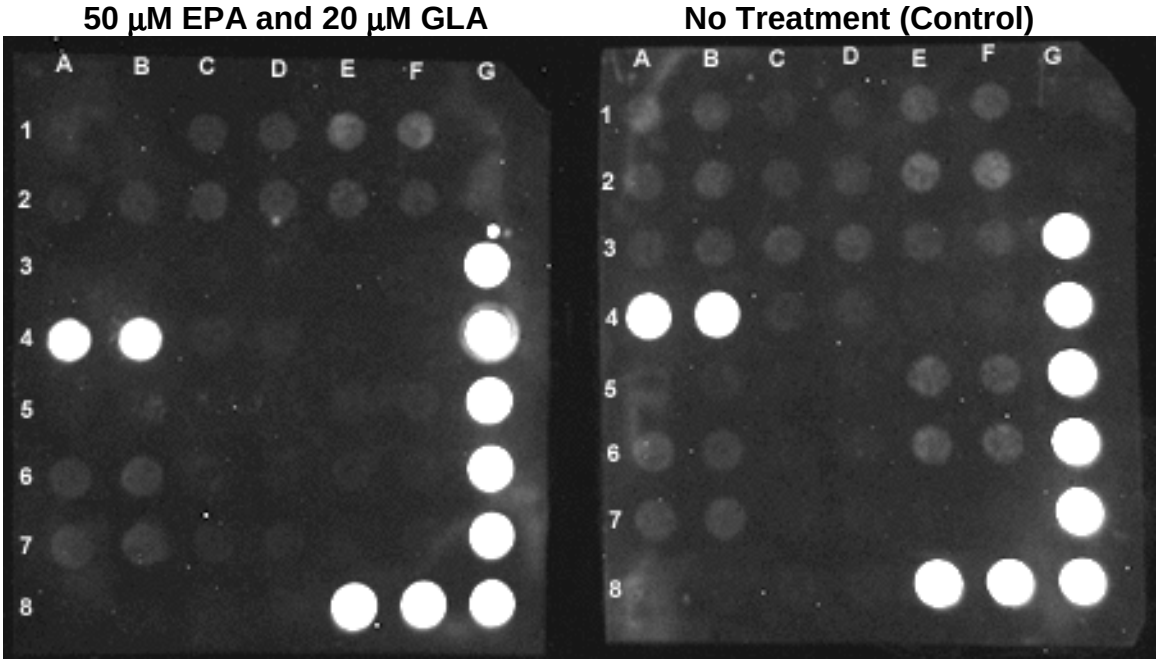

Figure 3.5.1. GEArray original series human apoptosis-2 gene arrays of 50 μM EPA and 20 μM GLA-treated and no treatment (control) HL-60 cells after 12-hour incubation.

Table 3.5.1. Apoptotic gene profile of EPA and GLA-treated HL-60 cells after 12-hour incubation.

50 μM EPA and 20 μM GLA-treated HL-60 cells			
Gene Name	GEA Location	Description	Intensity[1]
Bad	1A and 1B	Bcl2-antagonist of cell death	Absent
Bax	1C and 1D	Bcl2-associated X protein	Light
Bcl-2	1E and 1F	B-cell CLL/lymphoma 2	Moderate
Bcl-w	2A and 2B	Bcl2-like 2	Very light
Bcl-x	2C and 2D	Bcl2-like 1	Light
Caspase-1	2E and 2F	Apoptosis-related cysteine protease	Light
Caspase-10	3A and 3B	Apoptosis-related cysteine protease	Absent
Caspase-3	3C and 3D	Apoptosis-related cysteine protease	Absent
Caspase-5	3E and 3F	Apoptosis-related cysteine protease	Absent
Caspase-6	4A and 4B	Apoptosis-related cysteine protease	Heavy
Caspase-7	4C and 4D	Apoptosis-related cysteine protease	Very light
c-myc	4E and 4F	v-myc avian myelocytomatosis viral oncogene homolog	Absent
Fas	5A and 5B	Tumor necrosis receptor superfamily	Absent
Fas ligand	5C and 5D	Ligand for Fas	Absent
gadd 45	5E and 5F	DNA-damage-inducible transcript 1	Very light
gadd 45 β	6A and 6B	Homo sapiens growth arrest and DNA-damage inducible protein	Light
iNOS	6C and 6D	Inducible nitric oxide synthase (NOS)	Absent
mdm2	6E and 6F	Mouse double minute 2, human homolog of p53 binding protein	Very light
NFκB	7A and 7B	Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1	Light
p21Waf1	7C and 7D	Cyclin-dependent kinase inhibitor 1A	Very light
p53	7E and 7F	Tumor protein p53	Extremely light
DR5	8A and 8B	Homo sapiens TRAIL receptor 2	Absent
TRAIL	8C and 8D	Tumor necrosis factor (ligand) superfamily, member 10	Extremely light
β-actin	3G and 4G	Beta Actin	Heavy
GAPDH	5G, 6G, 7G, 8E, 8G, and 8F	Glyceraldehyde-3-phosphate dehydrogenase	Heavy
pUC18	1G and 2G	Bacterial plasmid	Absent

[1] Intensity is presented as a subjective measurement.

Table 3.5.2. Apoptotic gene profile of HL-60 cells after 12-hour incubation.

HL-60 cells (no treatment)			
Gene Name	GEA Location	Description	Intensity[1]
Bad	1A and 1B	Bcl2-antagonist of cell death	Light
Bax	1C and 1D	Bcl2-associated X protein	Very light
Bcl-2	1E and 1F	B-cell CLL/lymphoma 2	Light
Bcl-w	2A and 2B	Bcl2-like 2	Light
Bcl-x	2C and 2D	Bcl2-like 1	Very light
Caspase-1	2E and 2F	Apoptosis-related cysteine protease	Moderate
Caspase-10	3A and 3B	Apoptosis-related cysteine protease	Light
Caspase-3	3C and 3D	Apoptosis-related cysteine protease	Light
Caspase-5	3E and 3F	Apoptosis-related cysteine protease	Very light
Caspase-6	4A and 4B	Apoptosis-related cysteine protease	Heavy
Caspase-7	4C and 4D	Apoptosis-related cysteine protease	Extremely light
c-myc	4E and 4F	v-myc avian myelocytomatosis viral oncogene homolog	Absent
Fas	5A and 5B	Tumor necrosis receptor superfamily	Extremely light
Fas ligand	5C and 5D	Ligand for Fas	Absent
gadd 45	5E and 5F	DNA-damage-inducible transcript 1	Moderate
gadd 45 β	6A and 6B	Homo sapiens growth arrest and DNA-damage inducible protein	Light
INOS	6C and 6D	Inducible nitric oxide synthase (NOS)	Absent
mdm2	6E and 6F	Mouse double minute 2, human homolog of p53 binding protein	Moderate
NFκB	7A and 7B	Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1	Light
p21Waf1	7C and 7D	Cyclin-dependent kinase inhibitor 1A	Absent
p53	7E and 7F	Tumor protein p53	Absent
DR5	8A and 8B	Homo sapiens TRAIL receptor 2	Absent
TRAIL	8C and 8D	Tumor necrosis factor (ligand) superfamily, member 10	Extremely light
β-actin	3G and 4G	Beta Actin	Heavy
GAPDH	5G, 6G, 7G, 8E, 8G, and 8F	Glyceraldehyde-3-phosphate dehydrogenase	Heavy
pUC18	1G and 2G	Bacterial plasmid	Absent

[1] Intensity is presented as a subjective measurement.

Table 3.5.3. Relative intensities of apoptotic genes for control and EPA/GLA-treated HL-60 cells after 12-hour incubation.

Gene Name	GEA Location	Control Intensity[1]	EPA/GLA-treated Intensity[1]
Bad	1A and 1B	Light	Absent
Bax	1C and 1D	Very light	Light
Bcl-2	1E and 1F	Light	Moderate
Bcl-w	2A and 2B	Light	Very light
Bcl-x	2C and 2D	Very light	Light
Caspase-1	2E and 2F	Moderate	Light
Caspase-10	3A and 3B	Light	Absent
Caspase-3	3C and 3D	Light	Absent
Caspase-5	3E and 3F	Very light	Absent
Caspase-6	4A and 4B	Heavy	Heavy
Caspase-7	4C and 4D	Extremely light	Very light
c-myc	4E and 4F	Absent	Absent
Fas	5A and 5B	Extremely light	Absent
Fas ligand	5C and 5D	Absent	Absent
gadd 45	5E and 5F	Moderate	Very light
gadd 45 β	6A and 6B	Light	Light
INOS	6C and 6D	Absent	Absent
mdm2	6E and 6F	Moderate	Very light
NFκB	7A and 7B	Light	Light
p21Waf1	7C and 7D	Absent	Very light
p53	7E and 7F	Absent	Extremely light
DR5	8A and 8B	Absent	Absent
TRAIL	8C and 8D	Extremely light	Extremely light
β-actin	3G and 4G	Heavy	Heavy
GAPDH	5-7G, 8E, 8G, and 8F	Heavy	Heavy
pUC18	1G and 2G	Absent	Absent

[1] Intensity is presented as a subjective measurement.

Table 3.5.4. Percent of control for apoptotic genes in EPA and GLA-treated HL-60 cells after 12-hour incubation.

Gene Name	GEA Location	IDV[1] Control	IDV[1] EPA/GLA	% of Control[2]
Bad	1A and 1B	5315364	2909217	54.5
Bax	1C and 1D	3580386	3002469	83.6
Bcl-2	1E and 1F	5013522	4968123	98.8
Bcl-w	2A and 2B	4871190	2939892	60.2
Bcl-x	2C and 2D	4071186	3528852	86.4
Caspase-1	2E and 2F	6186534	3425784	55.2
Caspase-10	3A and 3B	4397568	2597559	58.9
Caspase-3	3C and 3D	4579164	2384061	51.9
Caspase-5	3E and 3F	4763214	2413509	50.5
Caspase-6	4A and 4B	72329196	29349840	40.4
Caspase-7	4C and 4D	3812289	2957070	77.3
c-myc	4E and 4F	3850326	2609829	67.6
Fas	5A and 5B	4196340	2923941	69.4
Fas ligand	5C and 5D	2984064	2527620	84.4
gadd 45	5E and 5F	5202480	3153390	60.4
gadd 45 β	6A and 6B	4961988	3409833	68.5
iNOS	6C and 6D	3023328	2346024	77.3
mdm2	6E and 6F	5384076	2793879	51.7
NFκB	7A and 7B	4617201	3801246	82.1
p21Waf1	7C and 7D	2909217	2374245	81.3
p53	7E and 7F	3631920	2768112	76.0
DR5	8A and 8B	4141125	1904304	45.8
TRAIL	8C and 8D	3063819	2413509	78.5
β-actin	3G and 4G	94627467	94931763	-

[1] IDV= Integrated Density Value

[2] % of control is = (IDV EPA/GLA gene/IDV β-actin EPA/GLA)-(IDV control gene/IDV β-actin control)/(IDV control gene/IDV β-actin control)*100+100

Discussion:

The hypothesis of this study was that treatment of HL-60 cells with EPA and GLA will increase the expression of pro-apoptotic genes and decrease the expression of anti-apoptotic genes as compared with control. Table 3.5.4 shows the integrated density value for control and EPA/GLA-treated HL-60 cells. This table shows that all genes were down-regulated as compared with control. This may be because HL-60 cells treated with EPA and GLA have increased rates of apoptosis as previously shown [3]. Increased rates of apoptosis are indicative of increased degradation of protein, DNA, RNA, and mRNA. It is possible that at 12-hours, the amount of intact mRNA, for the respective genes impregnated on the membranes, was degraded sufficiently enough to make it appear that all the genes had been down-regulated as compared with control when, in reality, there was most likely less mRNA present due to degradation not down-regulation.

Control HL-60 cells showed expression of Bad, Bax, Bcl-2, Blc-w, Bcl-x, caspases 1, 3, 5, 6, 7, and 10, Fas, gadd 45, gadd 45β, mdm2, NFκB, and TRAIL. EPA/GLA-treated HL-60 cells showed expression of Bax, Bcl-2, Bcl-w, Bcl-x, caspases 1, 6, and 7, gadd 45, gadd 45β, mdm2, NFκB, p21Waf1, p53, and TRAIL. The data for control cells agrees with previous reports that undifferentiated HL-60 cells express Bad, Bcl-2, Bcl-w, Fas, and caspases 1-4 and 7-10 [13;14]. Previous reports state that HL-60 cells have limited expression of caspase-6 [13]. This is a direct contradiction of the data presented here in where there was a heavy intensity of expression of caspase-6. Furthermore, reports have shown that other leukemia cell lines, including HL-60 cells, express

c-myc [15;16]. Our data indicates that HL-60 cells have no c-myc.

The expression of c-myc and Bcl-2 in leukemia cell lines has been linked to sensitivity and resistance to multiplication and death to exposure to long-chain polyunsaturated fatty acids (PUFA) [15]. Cells that express no Bcl-2 and low levels of c-myc were resistant to death by exposure to PUFA [15]. This does not agree with the data for our HL-60 cells. We have shown that PUFA induce apoptosis in HL-60 cells and our HL-60 cells do not appear to express c-myc [3]. Again, this is due to the late time point at which apoptosis was measured resulting in degraded mRNA and a loss of signal. Reports have shown that treatment of HL-60 cells with EPA and arachidonic acid (AA) does not reduce the levels of c-myc expression and that these fatty acids decrease proliferation and differentiation in HL-60 cells [16]. It is thought that c-myc is essential for several, but not all, types of apoptosis [16]. It has been demonstrated that exposure of T lymphocytes to dihomo-γ-linolenic acid (GLA is rapidly elongated to dihomo-γ-linolenic acid), EPA and AA result in a reduction of c-myc mRNA levels [17].

EPA has been shown to induce apoptosis in HL-60 cells with a decrease in expression of Bcl-2 that is greater than the decrease in expression of Bax [18]. The data from our study show an increase in the expression of Bcl-2 as compared with Bax in EPA/GLA-treated HL-60 cells after a 12-hour incubation. The reason for this is yet to be determined.

HL-60 cells share many functional characteristics with peripheral blood neutrophils, such as containing azurophilic granules, and are, therefore, considered the preferred immortalized cell line for the study of human neutrophil

responses *in vitro* [13;19]. Human neutrophils are short lived and will undergo apoptosis in 6-12 hours *in vitro*. HL-60 cells are an immortal cell line and do not spontaneously undergo apoptosis at an accelerated rate. Thus, HL-60 cells can be used measure induction of apoptosis by fatty acids and are a good model for peripheral blood neutrophils [19]. However, mature neutrophils express a different gene profile than HL-60 cells. For example, neutrophils essentially express no Bcl-2 [13;20]. Human neutrophils do not express the pro-apoptotic genes Bax, Bak, Bad, Bik, and Bcl-X_S, or the anti-apoptotic genes Bcl-X_L, and Bcl-2 [13;20;21]. Neutrophils express the pro-apoptotic genes Bak and Bad and the pro-apoptotic genes Bfl-a/A1 and Bcl-w [13;20]. The lack of Bcl-2 expression may account for neutrophils short life and rapid onset of apoptosis in 6-12 hours [13].

Human neutrophils express Fas but not Fas ligand [22]. This agrees with data determined in this study for HL-60 cells. The fact that neutrophils express the Fas death receptor makes neutrophils susceptible to apoptosis [22]. However, EPA/GLA-treated HL-60 cells did not express any Fas after 12-hour incubation. This again may be due to the overall decrease in viable mRNA due to rapid apoptosis and the mRNA levels may be below the limits of detection for the system used. Granulocyte macrophage colony stimulating factor (GM-CSF), which is elevated in the bronchoalveolar lavage fluid from patients with ARDS, has been shown to reduce the rates of Fas-mediated neutrophil apoptosis [22;23]. GM-CSF has also been shown to prevent apoptosis by the downregulation of pro-apoptotic Bax [20]. Furthermore, granulocyte colony

stimulating factor (G-CSF) is thought to delay neutrophil apoptosis by the upregulation of Bcl-X [24].

Our laboratory has previously shown that EPA and the combination of EPA and GLA induce apoptosis in HL-60 cells [3;11;12]. We demonstrated this using a variety of techniques including flow cytometry, apoptotic DNA laddering, TUNEL assay, and cleaved caspase-3 western blots. The EPA/GLA-treated HL-60 cells expressed no caspase-3 after 12-hour incubation. However, we recently showed, using western blotting techniques, that both pro-caspase-3 as well as active cleaved caspase-3 are present in no treatment and EPA-treated HL-60 cells after 12-hour incubation [12]. This strengthens our argument that the reason for the lack of expression of various pro-apoptotic genes as well as the overall down regulation as a percent of control is due not to a true down regulation but rather to a reduction of mRNA, via degradation caused by apoptosis, to levels below the sensitivity of the Super Array membranes. Gene expression at an early time point after exposure to EPA and GLA may leave enough intact mRNA to detect reliable differences in gene expression with Super Array membranes.

No conclusion can be made due to the unreliable data set generated in this experiment. However, the ability to elucidate the gene profiles in the future may enable us to develop new therapies in conjunction with dietary therapies that target specific genes to cause in the induction of neutrophil apoptosis thereby limiting tissue injury and resolving inflammation in patients with ARDS.

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Part 4

Conclusions

I. Conclusions

From the experiments conducted in this dissertation it is concluded, first, that eicosapentaenoic acid (EPA) and the combination of EPA and γ-linolenic acid (GLA) induce apoptosis and secondary necrosis in a time- and concentration-dependent manner in human promyelocytic HL-60 cells. This dissertation has also demonstrated that inhibitors of the lipoxygenase increase apoptosis in HL-60 cells treated with arachidonic acid (AA). The inhibition of the lipoxygenase enzymes, particularly 5-lipoxygenase, may shunt the metabolism of AA to other pathways involved in the regulation of HL-60 cellular apoptosis. Thus, downstream metabolic processing of AA by 5-lipoxygenase is critical in the regulation of HL-60 cell apoptosis. This dissertation has demonstrated similar effects when treating HL-60 cells with EPA and the combination of EPA and GLA. HL-60 cell death (cell death is apoptosis plus secondary necrosis) was increased in the presence of a 5-lipoxygenase inhibitor in the combination of EPA- and GLA-treated HL-60 cells. Conversely, as compared with the AA study, the inhibition of 12-lipoxygenase did not affect cell death in the combination of EPA- and GLA-treated HL-60 cells. Incubation with LTB_4 counteracted the effects of 5-lipoxygenase inhibition in the combination of EPA- and GLA-treated HL-60 cells in a concentration-dependent manner. These results indicate that LTB_4 is important for the maintenance of cell survival and suggests that the combination of EPA and GLA initiate cell death by a mechanism that likely involves reduced synthesis of LTB_4. Furthermore, this dissertation has

demonstrated that the increase in apoptosis and secondary necrosis induced by EPA is increased further with inhibitors of 5- and 12-lipoxygenase in HL-60 cells. Additionally, eicosanoid add-back experiments demonstrated that the 5-lipoxygenase products 5-HETE and LTB_4 and/or to a lesser extent the 12-lipoxygenase product 12-HETE are critical in the regulation of apoptosis and secondary necrosis in HL-60 cells. This study also demonstrated an increase in the amount of cleaved caspase-3 in HL-60 cells treated with EPA; implying that caspase-3 is involved in the apoptotic mechanisms induced by EPA. Finally, this dissertation determined that there are differences between the apoptotic gene profiles of no treatment and EPA/GLA-treated HL-60 cells. This indicates that EPA has an effect at the genetic level and not just at a metabolic level when inducing apoptosis in HL-60 cells.

This dissertation has helps to explain the observed effect of EPA and GLA in human and animal studies of acute respiratory distress syndrome (ARDS): i.e., that enteral nutrition with specialized diets containing EPA and GLA reduced the number of neutrophils and reduced the levels of LTB_4 in the bronchoalveolar lavage fluid, reduced pulmonary inflammation, and improved clinical outcomes in patients and animals with ARDS. This dissertation demonstrated that the observed clinical results may be due to increased neutrophil apoptosis induced by EPA and GLA. The induction of apoptosis likely occurs through a change in the eicosanoid profile as well a genetic change. Thus, the ability to alter the inflammatory cell phospholipids profile to reduce the amount of AA available for metabolism to pro-inflammatory eicosanoids, in conjunction with 5-lipoxygenase

inhibitory compounds, may lead to new therapies that will increase neutrophil apoptosis and provide a injury-limiting pathway in the resolution of pulmonary inflammation in ARDS.

Vita

Robert Clements Gillis was born in Baltimore, Maryland on March 28, 1975, and is the son of David J. and Katherine M. Gillis. Robert has two siblings, Kathleen and James. He received his high school diploma from St. Paul's School, in Brooklandville, Maryland, in June 1993. He then entered Gettysburg College in Gettysburg, Pennsylvania, majoring in Biology with a minor in Chemistry. He received a Bachelor of Science degree in Biology in May 1997. He then entered The University of Tennessee, Knoxville in August 1997, and began his graduate work in Animal Science on a teaching assistantship. Under the tutelage of Dr. R. N. Heitmann, he focused on animal nutrition and received his Master of Science degree in Animal Science in May 2000. He then entered the Comparative and Experimental Medicine program at The University of Tennessee on a research assistantship. Under the tutelage of Dr. M. D. Karlstad, he focused on physiology and pathophysiology and received his Doctor of Philosophy in Comparative and Experimental Medicine in August 2003. In August, 2003, Robert will begin to work on his Doctor of Medicine degree at Eastern Virginia Medical School in Norfolk, Virginia.