Effects of Dietary Polyunsaturated Fatty Acids and Oxylipins on Human EA.hy926 Endothelial Cells

by

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Abstract

Endothelial cells line the inner surface of all blood vessels and play a central role in maintaining vasculature homeostasis. Dietary polyunsaturated fatty acids (PUFAs) and their oxygenated derivatives, oxylipins, have been shown to modulate endothelial cell function in every aspect.

The overall hypothesis of this thesis is that dietary PUFAs and oxylipins affect the viability and proliferation of EA.hy926 human endothelial cells through a differential activation of mitogen-activated protein kinases (MAPKs) in growing and confluent (quiescent) states. Testing of this hypothesis was achieved by examining the effects of major PUFAs (linoleic acid [LA], arachidonic acid [AA], α-linolenic acid [ALA], eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) and the effects of lipoxygenase (LOX) inhibition on growing and confluent EA.hy926 human endothelial cell viability and proliferation, and MAPK-related cell signaling.

The results have shown that both substrate and growth state are important factors influencing the differential responses of endothelial cells to PUFAs. Specifically, cells became quiescent after reaching confluence when they were grown on Matrigel-coated plates, but not collagen-coated or plastic plates. AA, EPA, and DHA decreased the viability and proliferation rate of both growing and confluent cells in a dose-dependent manner, while LA and ALA had no effect. DHA induced apoptosis of confluent but not growing endothelial cells through a MAPK and caspase-3 dependent mechanism. Activation of MAPKs (p38, ERK1/2, JNK) by DHA followed a temporal sequence and was growth state-dependent. Treatment of the cells with PD146176 resulted in a reduction of viability in both growing and confluent cells, and a decline of proliferation in growing cells. However, these effects were not due to inhibition of 15-LOX,

the reported target of this compound. Rather, the oxylipin profile of PD146176-treated cells suggested CYP-derived oxylipins may mediate the cellular action of DHA. Interestingly, PD146176 operated through a p38MAPK, Akt and PPARα-dependent pathway.

In conclusion, the major finding of this thesis is that endothelial cell responses to PUFAs differ depending their growth state and presence of extracellular matrix. This may have significant clinical implications since dietary PUFA consumption could differentially affect healthy and dysfunctional endothelium, thus, influencing vascular homeostasis.

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List of Abbreviations

AA	arachidonic acid
7-AAD	7-aminoactinomycin D
ALA	α-linolenic acid
AP-1	activator protein 1
Ask1	apoptosis signal-regulated kinase 1
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma protein-2
BH	Bcl-2 homology
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCK-8	cell counting kit
CDC	cinnamyl-3,4-dihydroxy-α-cyanocinnamate
CDK	cyclin-dependent kinase
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A2
CRP	C-reactive protein
CV	cardiovascular
CVD	cardiovascular disease
CYP	cytochrome P450
DD	death domain

DGLA	dimoho-γ-linoleic acid
DHA	docosahexaenoic acid
DiHDoPE	dihydroxy-docosapentaenoic acid
DiHETrE	dihydroxy-eicosatrienoic acid
diHOME	dihydroxy-octadecenoic acid
DISC	Death Induced Signaling Complex
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
DR	death receptor
DUSP1	dual specific protein phosphatase 1
ECM	extracellular matrix
EIA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
EpDPE	epoxy-docosapentaenoic acid
EpDPE	epoxy-docosapentaenoic acid
EpETE	epoxy-eicosatetraenoic acid
EpETrE	epoxy-eicosatrienoic acid
EpETrE	epoxy-eicosatrienoic acid
EpODE	epoxy-octadecadienoic acid
EpOME	epoxy-octadecenoid acid
EpOME	epoxy-octadecenoic acid
ERK1/2	extracellular signal-regulated kinase 1/2
FA	fatty acid

FABP	fatty acid binding protein
$FABP_c$	cytosolic fatty acid binding protein
$FABP_{pm}$	plasma membrane associated fatty acid binding protein
FADD	Fas-associated protein with death domain
FATP	fatty acid transport protein
FBS	fetal bovine serum
FFA	free fatty acid
FFAR	free fatty acid receptor
FMD	flow-mediated dilatation
GC-MS	gas chromatography-mass spectrometry
GLA	γ-linoleic acid
GPCR	G protein coupled receptor
HDL	high density lipoprotein
HDoHE	hydroperoxy-docosahexaenoic acid
HDoHE	hydroxy-docosahexaenoic acid
HDoHE	hydroxy-docosahexaenoic acid
HEPE	hydroperoxy-eicosapentaenoic acid
HETE	hydroxy-eicosatetraenoic acid
HETE	hydroxy-eicosatetraenoic acid
HETE	hydroxy-eicosatetraenoic acid;
HETrE	hydroxy-eicosatrienoic acid
4-HHE	4-hydroxy-2-hexenal
4-HNE	4-hydroxy-2-nonenal

HHTrE	hydroxy-heptadecatrienoic acid
HMEC-1	human microvascular endothelial cell line 1
HODE	hydroperoxy-octadecadienoic acid
HOTrE	hydroperoxy-octadecatrienoic acid
HOTrE	hydroxy-octadecatrienoic acid
HpETE	hydroperoxy-eicosatetraenoic acid
HpODE	hydroperoxy-octadecadienoic acid
HRP	horseradish peroxidase
HSP	heat shock protein
IAP	inhibitor of apoptosis
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IP_3	inositol trisphosphate
IRS1	insulin receptor substrate 1
JNK1/2/3	c-Jun amino (N)-terminal kinase1/2/3
LA	linoleic acid
LC-MS	liquid chromatography-mass spectrometry
LDL	low density lipoprotein
LOX	lipoxygenase
LpL	lipoprotein lipase
LT	leukotriene
MA	mead acid
MAPK	mitogen-activated protein kinase

MAPKAPK2/3	MAPK activating protein kinase 2 and 3
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
MNK1/2	MAPK interacting protein kinase1/2
MSK1&2	stress-activated kinase 1&2
mTORC1	rapamycin complex 1
MUFA	monounsaturated fatty acid
NDGA	nordihydroguaiaretic acid
NEFA	non-esterified fatty acids
NO	nitric oxide
non-enz	non-enzymatic
NSAID	nonsteroidal anti-inflammatory drugs
oxLDL	oxidized low density lipoprotein
oxoETE	oxo-eicosatetraenoic acid
oxoODE	oxo-octadecadienoic acid
PAI-1	plasminogen activator inhibitor
PARP	Poly-ADP-ribose polymerase
PECAM-1	platelet endothelial cell adhesion molecule-1
PGI	prostacyclin
PGE	prostaglandin E
15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2

PI3K	phosphatidyinositide 3 kinase
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PPAR	peroxisome proliferator-activated receptor
PRAS40	proline-rich Akt substrate of 40 kDa
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
Rheb	Ras homolog enriched in brain
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SAPK	stress-activated protein kinase
Ser	serine
sEH	soluble epoxide hydrolase
SFA	saturated fatty acid
SMAC	direct inhibitor of apoptosis-binding protein with low isoelectric point
STAT1/3	signal transducer and activator of transcription
t-PA	tissue-type plasminogen activator
T2DM	type 2 diabetes mellitus
TF	tissue factor
TG	triglycerides
Thr	threonine
TNF-α	tumor necrosis factor-α
TRAIL-R	tumor necrosis factor-related apoptosis-inducing ligand receptor

triHOME	trihydroxy-octadecenoic acid
TSC2	tuberous sclerosis complex 2
TX	thromboxane
Tyr	tyrosine
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell
vWF	von Willebrand Factor
WPBs	Weibel Palade Bodies

Chapter 1: Literature Review

1.1 Overview

Endothelial cells are an important component of the vasculature. Dietary factors such as polyunsaturated fatty acids (PUFAs) can affect endothelial and vascular function. However, inconsistent results from dietary intervention studies suggest that PUFA modulation of endothelial cell properties is complex, and that the regulatory relationship between PUFAs and endothelial cells is not fully understood. The overarching question of this thesis is whether there are factors that could influence the effects the PUFAs on endothelial cells. This thesis proposed two factors, extracellular matrix and growth state, and examined how they influence cell viability and proliferation as well the molecular mechanisms mediating the effects of PUFAs on endothelial cells using an *in vitro* model. This thesis also sheds some light on the role of oxylipins (oxygenated derivatives and bioactive compounds derived from PUFA), specifically cytochrome P450 products on the alteration of endothelial cell viability and proliferation. Thus, the scope of this literature review is to provide background information and the current state of knowledge for understanding the central role of endothelial cells in the vasculature as well as the modulation of endothelial cell function by dietary PUFAs and oxylipins. Signaling pathways investigated in this thesis, including mitogen-activated protein kinase (MAPK), caspase, protein kinase B (Akt) and peroxisome proliferator-activated receptor (PPAR), are also reviewed.

1.2 Endothelial Cells

1.2.1 Endothelial Physiology

Endothelial cells form a single layer called the endothelium that covers the luminal surface of all blood vessels. Altogether, these cells function as an 'organ' to maintain homeostasis within the circulatory system. Besides serving as a physical barrier between the blood and underlying tissues, endothelial cells regulate many vital physiological processes such as inflammation, coagulation, and vascular tone [1-7]. Specifically, under normal conditions, endothelial cells 1) have selective permeability and help transport molecules; 2) produce anticoagulant factors such as nitric oxide (NO) and prostacyclin I_2 (PGI_2) to prevent platelet aggregation, as well as tissue-type plasminogen activator (t-PA) and urokinase that participate in fibrinolysis; and 3) secrete vascular relaxation factors such as NO and PGI_2 that act on vascular smooth muscle cells (VSMC) to regulate vascular tone and blood flow. Additionally, diverse physical, chemical and mechanical stimuli can transiently activate endothelial cells, and in this state the cells can 4) migrate and proliferate to assist with the repair of regions of a vessel that have been damaged, or to participate in vessel formation (namely, vasculogenesis and angiogenesis), and 5) participate in inflammatory responses by expressing adhesion molecules such as selectins, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1), and secreting pro-inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) to recruit inflammatory cells. Once these processes are complete, the cells will return to their normal inactive, quiescent and non-proliferative state, which is normally known as resolution.

1.2.2 Endothelial Dysfunction

If endothelial cell activation is not reversed, it will eventually lead to a dysfunctional state [8]. This typically occurs if the insult persists due to a chronic condition. Endothelium impairment (also referred to as endothelial dysfunction) is believed to be an early indication for the development of atherosclerosis [9, 10] and it has been shown to associate with cardiovascular (CV) events, specifically myocardial infarction and stroke [11]. Figure 1.1 illustrates the structural organization of the endothelium (Figure 1.1A) and the relationships between healthy, activated and dysfunctional endothelial cells (Figure 1.1B). In general, dysfunctional endothelial cells switch to a state characterized by proliferation, the release of fewer vasodilators, higher levels of pro-inflammatory cytokines, greater potential for thrombosis and platelet aggregation, and increased cell death [10]. Specifically, dysfunctional endothelial cells exhibit a decrease in the expression and activity of endothelial nitric oxide synthase (eNOS), decreased production and bioavailability of NO, increased production of reactive oxygen species (ROS) such as peroxynitrite and superoxide, increased production of adhesion molecules such as VCAM-1, ICAM-1, E-selectin and P-selectin, increased expression of chemokines such as MCP-1, decreased expression of the anti-coagulant factor t-PA, increased expression of pro-coagulant factors such as plasminogen activator inhibitor 1 (PAI-1) and von Willebrand Factor (vWF, an endothelium-derived glycoprotein, released when activated), and impaired endothelium barrier function. The levels of these molecules in conjunction with greater endothelial permeability, which can be assessed by microalbuminuria [12, 13], can be used to assess endothelial function. Circulating endothelial progenitor cells and endothelial microparticles levels can also be used to assess endothelial cell function *in vivo* [14].

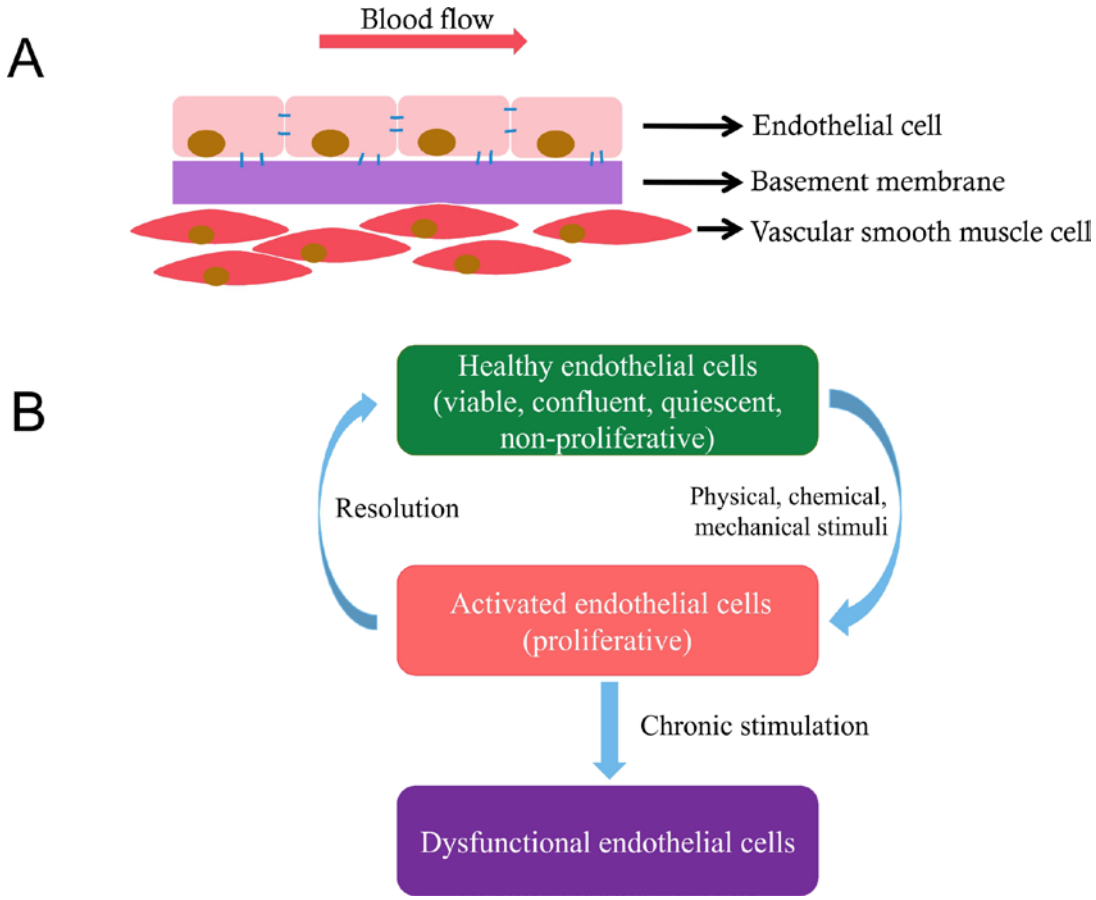


Figure 1.1 Structural organization of endothelium in blood vessel (A) and relationships among healthy, activated and dysfunctional endothelial cells (B).

1.2.3 Measurement of Endothelial Function

Endothelium-dependent vasomotion is a widely used marker to measure endothelial function non-invasively [14]. This is usually determined by measuring vasoactive substances such as NO, and their release in response to physiological or pharmacological stimuli. In parallel, endothelium-independent vasodilatory responses are used as controls for confounding factors. VSMC function can be tested by administration of nitroglycerine, an exogenous donor of NO [15], since this is the target tissue of endothelial cell-derived NO. Flow-mediated dilatation (FMD) is a surrogate marker for vascular function since it measures endothelium-dependent and NO-mediated arterial dilatation [16, 17]. In this procedure, blood flow is completely restricted by inflation of a cuff on the arm for 5 minutes. Ultrasound is then used to measure the change in brachial artery diameter (as a surrogate for conduit artery function) after restoration of blood flow from baseline. This restoration of blood flow increases shear stress on the endothelial cells and induces NO production, a process known as reactive hyperemia. The degree of change represents endothelial-dependent NO production. Although it is unusual for the brachial artery to develop atherosclerosis, the size of the brachial artery is similar to the coronary artery, and its vasodilator response correlates with coronary artery vasodilation [18]. Endothelium-independent vasodilation is measured by the same technique and calculation, but after sublingual administration of glyceryl trinitrate.

1.2.4 Endothelial Cell Lines

Ideally, primary endothelial cell cultures show the greatest similarity to vascular endothelial cells *in vivo* under both physiological and pathophysiological conditions, however, primary cells are usually only available from a limited number of sources, laborious to isolate, exhibit limited replication potential (a tendency to lose primary features and responsiveness after

5-10 passages) and lack consistency in response to the same treatment due to different donor origins [19]. Therefore, the generation and use of immortalized endothelial cell lines provides a more consistent system for studying the properties and responses of endothelial cells.

Various endothelial cell lines have been generated by virus infection, fusion with cancer cells, or transfection methodologies. One of the most frequently used and best characterized immortalized macrovascular endothelial cell lines is EA.hy926 cells. EA.hy926 cells were generated in 1983 by fusing human umbilical vein endothelial cells with the human lung carcinoma cell line A549 [20]. Weibel Palade Bodies (WPBs), which are the large rod-shaped organelles that are specific to endothelial cells, are considered the best marker for endothelial cells [21]. EA.hy926 cells have been shown to have WPBs, and express the typical characteristics of endothelial cells including the presence of cobblestone morphology at confluence, expression of vWF, ICAM-1, VCAM-1, E-selectin, tumor necrosis factor α (TNF-α), tPA, PAI-1, tissue factor (TF) [19]. EA.hy926 cells have also been used to study various aspects of endothelial cells including leukocyte adhesion [22], migration [23], angiogenesis [24] and barrier function[25]. The EA.hy926 cell line was selected for use in the project described in the thesis.

1.3 Extracellular Matrix and Basement Membrane

Basement membrane is a layered and structured extracellular matrix (ECM) located between the endothelium and the underlying smooth muscle cells [26, 27]; it is critical as it provides structural and biochemical support for the endothelial cells. Basement membrane is composed of two layers - basal lamina and reticular connective tissue, which consist of laminin, collagen, elastin, glycoproteins, proteoglycan, microfibrils, integrin, fibronectin, and mucopolysaccharides. Endothelial cells are responsible for the synthesis and deposition of some ECM components.

Adhesion of endothelial cells to ECM is required for cell viability, proliferation, migration, angiogenesis and blood vessel stabilization [28]. These various functions have been demonstrated as follows: 1) attachment of endothelial cells to ECM proteins such as vitronectin or fibronectin reduces endothelial cell apoptosis [29]; 2) expression and activation of cyclin-dependent kinases, which are required for cell cycle progression and therefore for cell proliferation, are dependent on endothelial cell adhesion to ECM; 3) interstitial collagen is highly effective at promoting haptotactic migration [28]; 4) degradation of basement membrane proteins by matrix metalloproteinase (MMP) is necessary for endothelial cell migration, invasion and angiogenesis; ECM also controls endothelial cell cytoskeleton in the process of angiogenesis through an integrin-dependent signal transduction pathway [28] and ECM scaffolds immobilize angiogenic cytokines and thereby coordinate signals transduced through both growth factor and ECM receptors; 5) integrity of junctions between endothelial cells, which also relies on anchorage of endothelial cells to ECM through integrins, is critical for maintaining a stabilized state of endothelial cells for normal functions.

1.3.1 Matrigel

Matrigel is a commercialized gelatinous mixture derived from Englebreth-Holm-Swarm mouse tumor cells [30]. It is rich in ECM proteins and commonly used as a basement membrane matrix for cells *in vitro* [31, 32]. Matrigel has been widely used to study angiogenesis/tube formation by endothelial cells [33].

1.4 Growth State

Under physiological conditions, the endothelium consists of a contiguous and confluent monolayer of endothelial cells that are in the quiescent state. Quiescence occurs due to contact inhibition, which leaves the cells in a viable but non-dividing state. However, quiescence is a reversible state that is different from irreversible cell-cycle arrest such as senescence and

terminal differentiation. Coller et al. [34] have shown that quiescence is an active state with up- and down-regulation of clusters of genes, which help maintain the cells in that state. Endothelial cells are able to exit quiescence and begin to proliferate in response to stimuli such as physical injury, pro-inflammatory cytokines, and angiogenic signals, which include growth factors and lipids like oxidized low density lipoprotein (oxLDL) [35, 36] and fatty acids [37]. Therefore, proliferation is active when endothelial cells are undergoing vascular repair, angiogenesis, or development of atherosclerosis when endothelial cells are migrating and proliferating to participate in the formation of plaque [38].

1.5 Cell Death

The human body is a multicellular organism, and while cell growth and differentiation are essential for normal growth and function of the body, so is cell death. Consequently, the balance between cellular growth and death has to be tightly regulated. If cell death ceases, a male human will end up with 2 tons of bone marrow and lymph nodes, and a 16 km long intestine [39]. Many diseases, like neurological disorders, cardiovascular disorders, and autoimmune disease, are related to a dysregulation of cell death, particularly apoptosis [40]. There are several ways a cell can die. The most recognized types of cell death are apoptosis, necrosis, and autophagy.

1.5.1 Apoptosis

Apoptosis a term that was first coined by Kerr et al. [41], and is a type of programmed autonomous cell dismantling which avoids eliciting inflammation. Apoptotic cells are characterized by morphological changes that include rounding-up of the cells, pseudopod retraction, a reduction of cellular volume, condensation of chromatin, nuclear fragmentation, formation of plasma membrane blebs and, in the later stages, loss of membrane integrity and engulfment by phagocytes [42]. Apoptosis depends upon activation of caspase, which in turn cleaves different substrates. Caspases are synthesized in a form of inactive zymogens, which can

be activated by cleavage of a pro-domain by other caspases [40]. There are two main pathways that result in caspase activation and thus apoptosis – extrinsic and intrinsic (Figure 1.2).

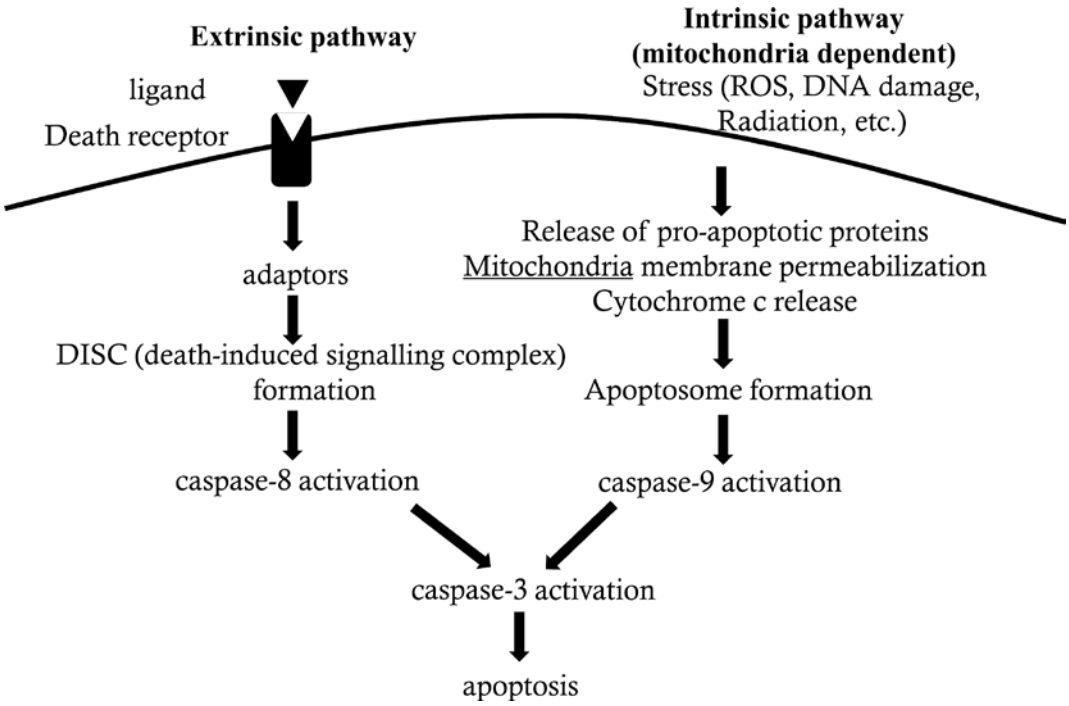


Figure 1.2 Illustration of extrinsic and intrinsic apoptosis pathways.

The extrinsic pathway is also called the death-receptor pathway because it involves ligands belonging to the TNF family that bind to the cell-surface death receptor (DR) family. Ligand binding to DRs causes a conformational change in the receptor, which leads to a recruitment of adaptor proteins such as Fas-associated protein with death domain (FADD) and TNF receptor-associated protein with death domain. This initiates the formation of a protein complex called Death Induced Signaling Complex (DISC), which has additional protein-protein interaction modules such as death effector domains that lead to the recruitment of initiator caspases such as caspase-8 and promotes their activation. The exact mechanism(s) for caspase-8 activation is still unknown, but some studies suggest it could occur through the interaction of caspase-8 with DISC that leads to their self-processing [43], dimerization [44] or conformational change in their

active site [45] resulting in their activation. Active caspase-8 further activates downstream caspases such as caspase-3, which is required for apoptosis. DRs belong to the TNF receptor gene superfamily, and have a cytoplasmic sequence named death domains (DDs). The most extensively studied DDs are Fas (CD95/APO-1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1/Death Receptor 4 [DR4]), and receptor 2 (TRAIL-R2/DR5/APO-2/KILLER) [46].

The intrinsic pathway processes through mitochondria and involves the B-cell lymphoma protein-2 (Bcl-2) family of proteins [40, 47]. Stresses such as increased intracellular ROS, deoxyribonucleic acid (DNA) damage, unfolded protein response, γ-radiation, and growth factor deprivation lead to increased mitochondrial permeability, which results in the cessation of adenosine triphosphate (ATP) production and the release of several proteins such as cytochrome c, apoptosis-inducing factor, direct inhibitor of apoptosis (IAP)-binding protein with low isoelectric point (also named as SMAC), from the mitochondria intermembrane space to the cytosol. Cytochrome c then interacts with apoptotic protease activating factor-1 and forms the apoptosome complex that subsequently recruits and activates caspase-9 through dimerization. Caspase-9 then activates downstream caspases such as caspase-3. The Bcl-2 family is characterized by possession of at least one Bcl-2 Homology (BH) domain. Pro-apoptotic proteins have one (e.g., Bcl-2-associated death promoter [Bad], Bik, Bid, Bim), or two to three (e.g., Bax, Bak) BH domains, while anti-apoptotic proteins have three or four BH domains (e.g., Bcl-2, Bcl-xl, Bcl-w). Pro-apoptotic proteins disrupt mitochondria integrity by binding to the voltage dependent anion channel or adenine nucleotide transporter and form pores in the membrane, while anti-apoptotic proteins interfere with the action of pro-apoptotic proteins [48].

The extrinsic and intrinsic pathways are not independent, since caspase-8 can cleave Bid, a pro-apoptotic protein, and generate truncated Bid that can permeabilize the mitochondria [49].

This process can facilitate cytochrome c release and links the two pathways together. Apoptosis is regulated by the Bcl-2 protein family as well as IAPs, which are endogenous inhibitors of apoptosis that can bind and inactivate caspase. However, SMAC could bind to IAP and inhibit its activity and allow apoptosis to proceed.

It is worth-mentioning that in the intrinsic pathway, apoptosis-inducing factor and endonuclease G relocate to the nucleus and mediate large scale DNA fragmentation. Concurrently, HTRP (high temperature requirement protein) A2 cleaves cytoplasmic substrates such as cytoskeleton proteins in a caspase-independent manner [50].

1.5.1.1 Importance of Endothelial Cell Apoptosis In the Vasculature

Although apoptosis means cell death, endothelial cell apoptosis plays an important role in the regulation and development of homeostasis in the body. Blood vessel remodeling and growth depends on the balance between cell apoptosis and proliferation. For example, during capillary development, macrophage-mediated endothelial cell apoptosis induces dying cells to block the capillary lumen, which subsequently leads to capillary regression [51, 52]. Both increased or insufficient apoptosis can result in various diseases including cardiovascular disease (CVD). For example, myocardial infarction involves myocardium apoptosis through the intrinsic pathway [53]. Apoptosis of endothelial cells has been detected in unstable coronary atherosclerotic plaque [54], and in areas where shear stress and flow are low [55]. Evidence has shown that lack of immune cell apoptosis contributes to autoimmune diseases [40]. Insufficient apoptosis also contributes to many cancers, which render them resistant to chemotherapies. Endothelial cells are responsible for supplying nutrients to tumor cells, and targeting tumor endothelial cells has received attention as a new therapeutic target for cancer treatment [56].

1.5.2 Necrosis

Unlike apoptosis, necrosis is characterized by the swelling of cells and the rupture of membranes resulting in the release of intracellular components [57]. Necrosis can be triggered by metabolic failure and rapid depletion of ATP, which usually occurs in ischemia [58, 59]. A series of events occurs during necrotic cell death, and this includes mitochondrial dysfunction, production of ROS, swelling of mitochondria, rapid depletion of ATP, loss of Ca^{2+} homeostasis, perinuclear clustering of mitochondria, lysosome rupture, activation of non-caspase proteases such as calpains and cathepsins, and eventually rupture of the plasma membrane [60]. Necrosis is not random and thus is a regulated process. The Poly-ADP-ribose polymerase (PARP) family of proteins is involved in the regulation of DNA repair, and the modulation of chromatin structure, transcription, replication and recombination [61]. PARP becomes activated by DNA strand breaks, which allows it to remove obstructing proteins thus enabling DNA repair to proceed [62]. When there is too much DNA damage for the cell to survive, PARP activation can reduce the available ATP. In the case of apoptosis, PARP is rapidly cleaved by caspase to preserve ATP stores so apoptosis can proceed. However, the cell shifts from apoptosis to necrosis when damage is extensive and the ATP stores become depleted. Increased intracellular Ca^{2+} also results in activation of proteases such as calpains and cathepsins that degrade cellular proteins [63].

1.5.3 Autophagy

Autophagy is characterized by the cellular degradation of cytoplasmic components such as non-essential or damaged sub-cellular structures through lysosomes, and subsequently, the release of digested products to the cytosol to recycle the macromolecules [64]. The major driving force for autophagy is starvation or deprivation of nutrients [65]; thus, autophagy is considered a

survival strategy, i.e. to delay cell death and not to destroy the cell. Studies have shown that deletion of key autophagic genes accelerates but does not inhibit cell death [42, 66]. However, if the degradation exceeds a certain limit, cells atrophy and die. There are three types of autophagy – macroautophagy, microautophagy, and chaperone-mediated autophagy [64]. The term autophagy is generally referring to macroautophagy.

Macroautophagy is a process in which there is formation of double-membrane phagophores that expand into large vesicles called autophagosomes that engulf part of the cytoplasm. This is followed by fusion of the autophagosome membrane with endosome and lysosome membranes, and the release of their contents into the autophagosome which digests the materials within. There are selective and non-selective autophagies. Selective macroautophagy targets damaged mitochondria, unwanted organelles and invasive microbes, while non-selective macroautophagy recycles random parts of the cytoplasm [67]. Regulation of autophagy involves the participation of insulin and amino acid signaling, Bcl-2, ROS, adenosine monophosphate-activated protein kinase, FADD, inositol trisphosphate (IP_3), calpin, and calcium [68].

Microautophagy involves the formation of autophagic tubes by invagination of lysosome membranes, and direct engulfing of that part of the cytoplasm [69]. Chaperone-mediated autophagy requires the transport of certain proteins, which have a specific targeting motif in their amino acid sequence, and the assistance of a cytosolic chaperone (heat shock cognate 71 kDa protein [HSC70]) and co-chaperones (carboxyl terminus of HSC70-interacting protein and heat shock protein 40 [HSP40], and HSP70-HSP90 organizing protein). This process brings the substrate to the lysosome surface via the receptor lysosome-associated membrane protein type 2A for internalization and subsequent degradation [70].

1.5.4 Other Cell Death Pathways

Besides apoptosis, necrosis and autophagy, other types of cell death have been identified and defined [42]. Examples include cornification, the formation of corneocytes (dead keratinocytes) in epidermis that contain an amalgam of specific proteins and lipids. Cornified skin is important by providing protection against mechanical force, and at same time, for stabilizing the structure. Anoikis is induced by detachment of cells from the ECM substrates, resulting in the loss of communication and nutrient uptake. Necroptosis is necrotic cell death dependent on receptor-interacting protein kinase-3, and it requires caspase-8 function to be inhibited or disrupted [71]. Ferroptosis is another type of cell death characterized by smaller mitochondria with condensed mitochondrial membrane densities, reduction of mitochondrial crista, rupture of outer mitochondrial membranes, and without condensation of chromatin [72].

1.6 Dietary Fatty Acids

1.6.1 Introduction

About 95% of dietary fat is consumed in the form of triglycerides (TGs) [73], which are composed of three fatty acids (FAs) attached to a glycerol backbone. The FAs in a TG can differ by the number of carbon atoms, degree of saturation, and location of the double bonds, and based on these factors are classified as either saturated FA (SFA), monounsaturated FA (MUFA), or PUFA [74]. Food and Agriculture Organization of the United Nations/World Health Organization has suggested classification of short chain FAs including 3 to 7 carbon atoms, medium chain FAs 8 to 13, long chain FAs 14 to 20, and very long chain FAs over 21 or more carbon atoms [75].

SFAs do not have double bonds. They represent 80-90% of total SFA intake from food; these fatty acids have strong atherogenic and thromobogenic properties [76].

MUFAs have one double bond. Representative MUFAs include oleic acid (C18:1n9), vaccenic acid (C18:1n7), and palmitoleic acid (C16:1n7). Both olive oil and canola oil are rich sources of MUFAs. Oleic acid was shown to have anti-atherogenic and anti-thrombotic activity, and increase the high density lipoprotein (HDL)/low density lipoprotein (LDL) ratio [76], while olive oil has been shown to be protective against carcinogenesis [77].

The PUFA family is separated into n-6 PUFA and n-3 PUFA depending on the location of first double bond from the methyl end. N-6 PUFAs have their first double bond located on the sixth carbon from the methyl end. The precursor of the n-6 PUFAs is linoleic acid (LA, C18:2n6). LA, the predominant fatty acid in conventional sunflower oil and soybean oil, can be converted into γ-linoleic acid (GLA, C18:3n6), dihomo-γ-linoleic acid (DGLA, C20:3n6), arachidonic acid (AA, C20:4n6) and docosatetraenoic acid (C22:4n6). The major n-3 PUFAs, which have their first double bond on the third carbon from the methyl end of the hydrocarbon chain, consist of α-linolenic acid (ALA, C18:3n3), eicosapentaenoic acid (EPA, C20:5n3), docosapentaenoic acid (DPA, 22:5n3) and docosahexaenoic acid (DHA, 22:6n3). ALA is the dietary essential precursor of all n-3 PUFAs, and is rich in plant sources such as flaxseed and flaxseed oil [78]. EPA, DPA and DHA make up a large portion of the n-3 PUFAs in marine animals, such as fatty fish, and they can be derived from ALA. The conversion rate of ALA into EPA and DHA is very low in humans based on tracer studies utilizing the blood compartment [79]. LA and ALA are considered to be essential FAs because they cannot be synthesized in the human body and have to be obtained from the diet due to a lack of Δ12- and Δ15-desaturases.

In the bloodstream, fatty acids circulate in two major forms: as non-esterified fatty acids (NEFA) associated with albumin, or as esterified fatty acids (constituents of TG, phospholipids and cholesterol esters) and packaged in lipoproteins. NEFAs are also called free fatty acids

(FFAs). The fatty acids in NEFA and lipoproteins can be from dietary or endogenous sources. During fasting, FFAs are generated from the white adipose tissue through a process called lipolysis and enter the bloodstream where they are non-covalently bound to plasma albumin. They exist in relatively low concentrations in healthy individuals under fasting condition (300 μM) [80], but are elevated to 900 μM in the postprandial state and in obese individuals [81]. High NEFA levels are also shown to have an association with increased CVD risk and mortality [82, 83], as well as impact endothelial functions such as apoptosis, NO production and vasodilation [84-88]. Circulating endogenous or dietary FFAs taken up by the liver can be used for TG synthesis and packaged into very low density lipoproteins for delivery to the tissues, whereas chylomicrons are produced in the intestinal tract and contain dietary fatty acids.

Fatty acids have many physiological roles, both structurally and functionally [89]. Fatty acids in the form of phospholipids make up the core structure of the phospholipid bilayer of plasma membranes. The composition of fatty acids also determines membrane fluidity, which has important roles in the regulation of membrane associated protein activity such as enzymes, receptors, and ion channels [90, 91]. Fatty acids also serve as energy substrates in the form of TGs. Meanwhile, fatty acids are involved in cellular signaling and act as ligands of nuclear receptors or transcription factors that influence gene expression [92]. Fatty acids are also the precursors of lipid mediators, originally known as eicosanoids (including prostaglandins, leukotrienes and thromboxanes) and now known as oxylipins (will be introduced in the following section), which regulate inflammation, coagulation, and vasoactivity [89].

1.6.2 Interaction Between Fatty Acids and Endothelial Cells

Circulating fatty acids have to pass through the endothelium to reach different cell types and tissues. However, how dietary lipids are transported through the endothelium to be utilized by peripheral tissues is still poorly understood.

The interaction between lipoprotein and endothelium begins with lipoprotein lipase (LpL), which is anchored on the surface of endothelial cells. LpL is sysnthesized by parenchymal cells in adipose tissue, cardiomyocytes, skeletal muscle cells, macrophages and mammary gland cells, and secreted into the interstitial fluid. From there, it diffuses to the luminal surface of the capillary endothelium and becomes bound through its interaction with heparin sulfated proteoglycans [93] and glycosyl inositol HDL binding protein 1 (a specific LpL binding protein) [94]. In this location, LpL hydrolyzes TG-rich lipoproteins (a lipolytic process), which results in the release of FFAs [95]. Increased LpL activity is associated with lower circulating TG levels in humans [96] and, in fact, the amount of LpL on the surface of endothelial cells is highly dependent on the circulating TG levels [97]. The activity of LpL is also tightly regulated at the transcriptional and post-translational levels to ensure the prompt clearance of TG in the bloodstream in relation to the tissue demand for fatty acids [98].

FFAs resulting from release from lipoproteins or NEFAs are quickly taken up by endothelial cells and subsequently allowed to penetrate through the arterial wall and reach the muscle and adipose tissue. Initially, it was thought that FFAs enter the plasma membrane of endothelial cells through just passive diffusion [99], however, more studies support the view that this translocation is protein-mediated [100]. In fact, uptake of FFAs is modulated by the cell surface scavenger receptor CD36 [101], several G protein coupled receptors (GPCRs), fatty acid transport protein (FATP), and intracellular fatty acid binding proteins (FABPs).

CD36 is also called fatty acid translocase and is expressed in a variety of cell types such as endothelial cells, monocytes, platelets and the parenchymal cells of adipose tissue and muscle [102]. CD36 acts an acceptor for fatty acids at the outer side of membrane. Once fatty acids have entered the inner side of the membrane, CD36 facilitates their further transport by providing a docking site for cytosolic FABP ($FABP_c$) [103] or for enzymes such as acyl-CoA synthetase to form acyl-CoA esters [104].

Ligands for FFA receptors (FFARs) depend on the chain length of the fatty acids; generally, short-chain FFAs activate FFA2 (GPR43) and FFA3 (GPR41), medium- and long-chain FFAs activate FFA1 (GPR40) and FFA4 (GPR120), and medium-chain FFAs also activate GPR84 [105]. FATPs are a family consisting of six large multi-transmembrane spanning proteins (FATP1-6) [106]. FATP3 and FATP4 play important roles for the uptake of LCFA by endothelial cells. Recent studies showed that regulation of fatty acid transport to the myocyte involves vascular endothelial growth factor B, which stimulates expression of FATP3 and FATP4 on the endothelial cell surface [107]. FABPs consist at least 13 members that work as intracellular FA handling proteins [108]. There are $FABP_c$ and plasma membrane associated FABP ($FABP_{PM}$). FFAs bind to $FABP_c$ after they have entered the inner side of plasma membrane. $FABP_{PM}$ assists CD36 in FFA translocation by accepting FFA at the cell surface, which increases the local concentration.

Because of their unique locations, endothelial cells are in constant contact with circulating fatty acids. According to the lipotoxicity hypothesis, lipids will be deposited in myocytes and hepatocytes when the adipose tissue is overloaded [109]. However, endothelial cells are not only responsible for translocating fatty acids to other tissues or organs, but also under certain circumstances, store lipids in the form of lipid droplets. Lipid droplet formation in endothelial

cells is a new area of interest and has not been well studied. Lipid bodies were first observed in bovine aortic and pulmonary arterial endothelial cells under hypoxic conditions [110]. Majzner et al. [111, 112] found AA and EPA could induce lipid droplet formation in primary human aortic endothelial cells and a human microvascular endothelial cell line (HMEC-1). Kuo et al. [113] gavaged a single bolus of olive oil to overnight fasted mice, and observed a maximum TG accumulation in blood 90 min after gavage, which returned to baseline after 180 min. However, lipid droplet formation in endothelial cells of the thoracic aorta appeared at 180 min after gavage, and disappeared after 270 min. Therefore, they hypothesized that the endothelium works as a fat buffering system by storing excess FFAs in the form of lipid droplets to protect endothelial cells from fatty acid-induced ER stress and apoptosis. Astanina et al. [114] identified lipid droplets in HMEC-1 that were more than a lipid storage pool; they identified these lipid droplets as functional organelles that existed in tunneling nanotubes, structures which may facilitate intercellular communications.

Additionally, fatty acids mediate their effects in endothelial cells via nuclear receptors such as the PPARs. PPARs, members of the nuclear receptor superfamily, are also transcription factors. They bind DNA and at same time, regulate gene expression. There are three PPAR subtypes (PPARα, PPARβ/δ, PPARγ) in vertebrates and they are important mediators of inflammation, lipid metabolism, cell growth and differentiation [115, 116].

1.6.3 Dietary Polyunsaturated Fatty Acids and Endothelial Cell Function

Evidence from intervention studies has shown that dietary PUFA supplementation can modulate endothelial cell function [117, 118]. Most research focuses on the influence of diet on health over long periods of time, however, postprandial and non-fasting TG concentrations are

independent risk factors for CVD, thus rendering the importance of examining single meal effects on vascular health [119, 120].

FMD is a widely used non-invasive method for assessing vascular function after single meals or chronic interventions. Although a single meal rich in n-6 PUFA does not induce a change in FMD in postmenopausal women [121], several long-term n-6 PUFA intervention studies (>4 weeks) showed improvements in FMD in children with familial hypercholesterolemia or familial combined hyperlipidemia [122], and in patients with metabolic syndrome [123]. On the other hand, several studies have reported no change in FMD after long-term intervention with n-6 PUFA (LA) in hypercholesterolemic subjects [124, 125] or otherwise healthy persons with moderate hypertriglyceridemia [126].

Studies on the single meal effects of n-3 PUFA (EPA+DHA) have reported that FMD was not changed in healthy subjects [127] or in coronary artery disease patients [128], but FMD was increased in a randomized crossover study of healthy and hypercholesterolemic subjects [129] who received approximately 5.4 g of ALA daily in the form of walnuts added to a Mediterranean-style diet for one week. It is noteworthy that the latter study [129] found that FMD was unchanged in healthy subjects and increased in hypercholesterolemic subjects after the walnut meal. It is interesting that the effect of n-3 PUFA on FMD also depends on the lipid profile of the subject, as there was an impact (increased FMD) in subjects with high fasting TG levels, but not in healthy subjects.

Many studies have examined the chronic effect of n-3 PUFA on FMD and report an improvement of FMD after the intervention compared to baseline in patients with moderate hypercholesterolemia, primary hypertriglyceridemia, familial hypercholesterolemia and familial combined hyperlipidemia, postmenopausal women with moderate hypertriglyceridemia,

postmenopausal women with peripheral artery disease, type 2 diabetes mellitus (T2DM) patients with established atherosclerotic CVD and without prior CVDs, healthy offspring of T2DM patients, patients with acute myocardial infarction and successful percutaneous coronary intervention with stent implantation, patients with systemic lupus erythematosus, healthy smokers, healthy male cyclists with endurance training for 7 years, and middle aged men and women with metabolic syndrome [122, 124, 125, 130-139]. However, FMD was not improved by n-3 PUFA supplementation in several studies where adult subjects had HIV-infection and moderate CVD risk [140], or subjects had moderate hypertriglyceridemia and T2DM [126] and were being treated for hypertension [141, 142]. Similar to the single meal effect studies, n-3 PUFA for several weeks did not improve FMD in healthy subjects in three studies [143-145].

Overall, studies investigating effects of n-6 PUFA on endothelial cell function are still not be able to verify their positive or negative impact, but evidence for potential benefits of n-3 PUFA on vascular health is becoming more compelling. Specifically, n-3 PUFA exert beneficial effects in patients with a concurrent disease in both single-meal studies and long-term interventions, possibly to counterbalance the adverse effects of the disease condition, but n-3 PUFA supplementation has no effect on vascular function in healthy individuals.

1.6.4 Mechanisms for Dietary n-3 PUFA Regulation of Endothelial Cell Function

Correlations between change of FMD (difference between baseline and end of study) and other parameters in n-3 PUFA interventions studies may indicate some potential mechanisms of action for explaining how n-3 PUFA may modulate endothelial cell function. Change of FMD has been inversely correlated with plasma concentrations of C-reactive protein (CRP) [124], TG [134], and TNF-α [134] as well as the cholesterol-to-HDL ratio [146]. n-3 PUFA intake was also correlated with changes in plasma lipids (HDL-cholesterol and TG) [147], changes in

inflammatory status (based on CRP and TNF-α levels) [148], improvements in endothelial function [149], and increased EPA/DHA content in platelet membranes (which could possibly be affecting the ion channels and enzyme activities) [136].

The mechanism(s) for how n-3 PUFAs could benefit endothelial cell function are not fully understood, however, *in vitro* studies have provided us with considerable evidence for how n-3 PUFA could directly affect endothelial cell function and cell status. The actions of n-3 PUFA on endothelial cells are believed to operate through the regulation of NO production [149, 150], vasodilation/vasoconstriction [151, 152], inflammation [153-157], oxidative stress [158], the renin-angiotensin system [159-161], alteration of blood lipid profiles [125, 134, 146], modulation of oxylipin profiles [160, 162-169], and as ligands of nuclear receptors and transcription factors that regulate gene expression, such as PPAR-α, -β/δ, -γ1 and -γ2, liver X receptors type α and β, hepatic nuclear factor-4α, sterol regulatory element binding protein-1 and -2, and carbohydrate regulatory element binding protein/Max-like factor X [89].

1.7 Oxylipins

Oxylipin refers to the oxygenated products of PUFAs that are generated through three enzymatic pathways – cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP). COX has two isoforms, COX-1 and COX-2. COX-1 is universally expressed in most tissues, while COX-2 is constitutively expressed in placenta, macula densa of the kidney and brain, and only expressed in other tissues under certain physiological stimuli [170, 171]. COX converts PUFAs into prostaglandins, and thromboxanes, which play important roles in inflammation, cancer, development, etc [170, 172]. Inhibition of COX with inhibitors such as aspirin, indomethacin and other nonsteroidal anti-inflammatory drugs (NSAIDs) reduces inflammation and platelet aggregation, but since it also inhibits production of protective

prostaglandins (PGI_2 and prostaglandin E_2 [PGE_2]) by COX-2, it creates side effects such as gastrointestinal ulceration, bleeding, and perforation [173, 174].

Major mammalian LOX enzyme families include 5-, 12-, and 15-LOX [175]. These enzymes were named so because they were initially shown to insert oxygen at carbon 5, 12, or 15 of AA and produce 5-, 12- or 15-hydroperoxy-eicosatetraenoic acid (5-, 12-, or 15-HpETE), which can be further reduced to 5-, 12-, or 15-hydroxy-eicosatetraenoic acid (5-, 12-, or 15-HETE) by glutathione peroxidase [176-178]. LOX also catalyzes the formation of leukotrienes as well as hydroperoxy- products from other PUFAs, namely, hydroperoxy-octadecadienoic acid (HODE), hydroperoxy-octadecatrienoic acid (HOTrE), hydroperoxy-eicosapentaenoic acid (HEPE) and hydroperoxy-docosahexaenoic acid (HDoHE) from LA, ALA, EPA and DHA, respectively, and then to their hydroxy-products.

CYP is a superfamily of enzymes that includes more than 50 isoforms produced by 57 P450 genes in humans [179]. The class name of these enzymes stems from its characteristic spectrophotometric absorbance at wavelength 450 nm when reduced and bound by carbon monoxide. Besides producing oxylipins, CYP enzymes are also involved in biosynthesis and metabolism of vitamin D, bile acid, cholesterol, steroid hormones, etc [180]. Regarding the production of oxylipins, CYP epoxygenase converts LA, AA, ALA, EPA and DHA into epoxy-octadecenoid acid (EpOME), epoxy-eicosatrienoic acid (EpETrE), epoxy-octadecadienoic acid (EpODE), epoxy-eicosatetraenoic acid (EpETE), and epoxy-docosapentaenoic acid (EpDPE), respectively, while CYP ω-hydroxylase converts LA, AA, EPA and DHA into hydroperoxy-octadecadienoic acid (HpODE, can be also produced by LOX), HETE, HEPE and hydroxy-docosahexaenoic acid (HDoHE), respectively.

Generation of oxylipins starts once the precursor PUFA is released from sn-2 position of the glycerol backbone of a membrane phospholipid by cytosolic phospholipase A_2 ($cPLA_2$) [181]. Eicosanoids, which are twenty-carbon oxygenated fatty acids derived from AA, were the first class of oxylipins to be discovered and studied [182, 183]. The eicosanoid family consists of products such as prostanoids (includes prostaglandins and thromboxanes), leukotrienes, and HpETEs. Oxylipins generated from eighteen-carbon and twenty-two-carbon fatty acids are named octadecanoids and docosanoids, respectively. Examples of octadecanoids include hydroxy/hydroperoxy/dihydroxy/trihydroxy-octadecenoic acid and examples of docosanoids include hydroxy/hydroperoxy/epoxy/dihydroxy-docosahexaenoic acid.

Oxylipins are synthesized by nearly all mammalian cell types and released rapidly to the extracellular space in response to different stimuli. Oxylipins are found in all tissues, and biological fluids like urine and blood [184]. Pathways and metabolism of oxylipins from various PUFAs can be found in the review by Gabbs et al. [184].

Studies of the impact of oxylipins on endothelial cells are now emerging, although the initial studies were focused on the interaction of immune cells such as leukocyte and platelets with endothelial cells [185-191] due to the important roles of eicosanoids in inflammatory responses. Now our knowledge has expanded to show they can modulate every aspect of endothelial cell function including cell survival and apoptosis [192, 193], proliferation [194, 195], migration [196, 197], barrier function [198-203], vasorelaxation/vasoconstriction [204, 205], and angiogenesis [195, 206, 207].

The actions of oxylipins are mediated by membrane associated G-protein-linked receptors, which are coupled to a range of intracellular signaling pathways, such as adenylyl cyclase and phosphatidylinositol, causing subsequent changes in second messengers such as cyclic adenosine

monophosphate (cAMP) and IP_3 [208], or nuclear receptors such as PPARs [176, 209]. Some of the known receptors include D types, E types, F types, I types for prostaglandins [210], TP for thromboxanes [211], LT for leukotrienes [212], and ALX for lipoxins [213], however, the receptors for other oxylipins and how they operate remains unknown. Oxylipins may share the same receptors as fatty acids or there may be other receptors yet to be discovered.

Oxylipins are now believed to be one of the lipid mediators of PUFAs. Direct evidence in support of this concept is that oxylipins such as eicosanoids that have been well characterized with respect to the inflammatory response, and they interact with immune cells, VSMCs, and endothelial cells to modulate cell trafficking and adhesion, angiogenesis, vascular permeability, and vasomotion [214]. Indirect evidence for oxylipins being the lipid mediators of PUFAs is that intervention studies which modify dietary fatty acid composition show a correlation between changes of endothelial cell function and oxylipin profiles [160, 162-169].

Oxylipins have very similar structures and chemical characteristics, thus making their separation and detection challenging [215]. Methods for analyzing oxylipins have also evolved from enzyme-linked immunosorbent assays (EIA) [216, 217], to gas chromatography-mass spectrometry (GC-MS) [218], and liquid chromatography-mass spectrometry (LC-MS) [219]. However, EIA requires a specific antibody for each oxylipin, and therefore makes the method inefficient due to a lack of specificity and the ability to detect multiple analytes in a single analysis since few antibodies for oxylipins are commercially available. GC-MS was an improved method because it has overcome the limitations of EIA, but it requires chemical derivatization of the compounds to improve their volatility. Even so, no single derivatization method works for all oxylipins and Murphy et al. [220] have found that some eicosanoids are not suited for GC-MS. Several LC-MS-based methods have been developed and these enable large-scale and high-

throughput analysis of oxylipins from small volumes of biological samples [215, 219, 221, 222]. Specifically, development of electrospray ionization allows analysis of oxylipins in an aqueous environment, and high performance LC permits isolation based on their chemical and physical properties. Collision-induced decomposition creates characteristic precursor and product transitions that can be detected on the MS. Combining these methods enables the high-throughput detection of oxylipins.

1.8 Cell Signaling

1.8.1 MAPK

Mitogen-activated protein kinases (MAPKs) are protein Serine (Ser)/Threonine (Thr) kinases that transduce signals from the extracellular space into the intracellular space through membrane-embedded receptors such as receptor tyrosine kinases (RTK), GPCRs, and toll-like receptors, and regulate a variety of cellular responses including cell survival, apoptosis, proliferation, migration, differentiation, and gene expression [223]. The traditional and most widely studied MAPKs include p38 isoforms (α, β, γ, and δ), extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun amino (N)-terminal kinase1/2/3 (JNK1/2/3). Each MAPK pathway is comprised of three sequentially and independently acting kinases, which consists of a MAPKK kinase (MAPKKK or MAP3K or MEKK), MAPK kinase (MAPKK or MEK), and MAPK. Each MAPKKK is a protein Ser/Thr kinase, which is activated through an interaction with a small GTP-binding protein of the Ras/Rho family. Subsequently, each MAPKKK phosphorylates and activates its specific MAPKK, and these in turn phosphorylate Thr and tyrosine (Tyr) residues of MAPK, which activates the MAPK.

1.8.1.1 p38 MAPK

p38α is universally expressed in all cell types, while p38β, p38γ and p38δ have specific expression patterns. For example, brain, skeletal muscle, and endocrine glands have higher levels

of p38β, p38γ and p38δ, respectively [224]. p38 MAPK generally responds to stress stimuli [225, 226], especially p38α which mediates inflammatory processes in response to stimuli such as cytokines, chemokines, and lipopolysaccharide by targeting nuclear transcription factors such as NF-κB, thus affecting NF-κB target gene activities [224, 227]. p38 is also involved in the regulation of cell survival and proliferation by targeting the Bcl-2 family, cyclins, and cyclin-dependent kinase (CDK) [228, 229].

The two main MAPKKs for p38 MAPK are MKK3 and MKK6, although a variety of other MAPKKKs, such as MEKK4 [230-232], ASK1/MAPKKK5 [233], DLK/MUK/ZPK [230, 234] and TAK1[235], can participate in the activation of p38 under specific conditions. Substrates of p38 MAPK include mitogen and stress-activated kinase 1&2 (MSK1&2), which can directly phosphorylate and activate transcription factors such as cAMP response element-binding protein, activating transcription factor 1, NF-κB, signal transducer and activator of transcription (STAT1/3) as well as proteins located in the nucleosome such as histone H3 and high-mobility group protein 14. p38 MAPK substrates also include MAPK activating protein kinase 2 and 3 (MAPKAPK2/3), which subsequently activate HSP27 (important for actin filament remodeling), eukaryotic elongation factor 2 kinase (important for elongation of mRNA during translation), and MAPK interacting protein kinase1/2 (MNK1/2) which phosphorylates eukaryotic translation initiation factor 4E [224, 236]. Activation of p38 MAPK plays an important role in inflammation, apoptosis, cell cycle control, differentiation, senescence and tumor suppression [236]. p38 MAPK activation is tightly regulated by its upstream MAPKK and MAPKKK, and negatively regulated by members of the MAP kinase phosphatase (also named dual-specificity phosphatase [DUSP]) [237] and other of phosphatases such as serine/threonine protein phosphatase type 2C [238, 239]. DUSPs play important roles in determining the duration and

magnitude of the MAPK activities, and they vary in substrate specificity, tissue distribution and cellular localization [240].

1.8.1.2 Extracellular Signal-regulated Kinase1/2 (ERK1/2)

ERK1/2 can be activated by growth factors such as epidermal growth factor, nerve growth factor, and platelet-derived growth factor [241]. The ERK1/2 module includes MAPKKK (A-Raf, B-Raf and Raf-1) and MAPKK (MEK1 and MEK2) [223]. ERK1/2 modules are typically located in the cytoplasm and translocate to the nucleus after stimulation [242, 243]. ERK1/2, upon activation, can phosphorylate a variety of substrates including MSK and MNK, which are also substrates of p38 MAPK, as well as other substrates such as the ribosomal S6 kinase family, which plays important roles in cell survival and motility [244]. Different from p38 MAPK, ERK1/2 is important in the regulation of cell proliferation. Sustained activation of ERK1/2 is a prerequisite for cell entry into the cell cycle and for transition from G_1 to S phase [245]. ERK1/2 also stabilizes c-Fos protein, which facilitates its interaction with c-Jun to form the activator protein 1 (AP-1) complex, and subsequently induce expression of cell cycle proteins such as cyclin D1, and allows cell cycle progression [246].

1.8.1.3 C-Jun N-terminal Kinase (JNK)

JNK got its name when it was found to bind to specific regions within the c-Jun transactivation domain and cause phosphorylation of this protein [247]. JNK has three isoforms – JNK1-3 (also known as stress-activated protein kinase [SAPK]α, β, and γ) [248]. JNK1 and JNK2 are expressed in most cell types, while JNK3 is located primarily in cardiac myocytes, testis and neurons [249]. JNK was also known as SAPK as it responds to stresses such as UV light, oxidative stress, heat shock, cytokines, DNA and protein synthesis inhibitors, as well as, but to a lesser extent, growth factors [250]. The JNK module includes MAPKKK (MEKK1-4,

MLK1-3, DLK, TAO1/2, TAK1, ASK1/2 and Tpl-2) [251, 252] and MPKKK (MKK4 and MKK7) [253]. Similar to ERK and p38 MAPK, upon activation, a proportion of JNK translocates from the cytoplasm to the nucleus, and subsequently activates substrates such as the transcription factor c-Jun, which promotes AP-1 complex formation, and regulates cell proliferation [254, 255]. Substrates of JNK also include p53, ATF-2, nuclear factor of activated T-cells - cytoplasmic 1, STAT3, and JunB (a transcription factor, AP-1 subunit) [250, 256]. JNK has also been implicated in the regulation of apoptosis [257] and differentiation [258].

1.8.2 Akt

Ligands such as growth factors, cytokines, and insulin bind to receptors containing RTKs that stimulate the recruitment of class I phosphatidyinositide 3 kinase (PI3K) to the plasma membrane. PI3K is activated through direct binding or through tyrosine phosphorylation of scaffolding adaptors such as insulin receptor substrate 1 (IRS1), which bind and activate PI3K. PI3K then phosphorylates glycerophospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3) [259]. Both 3-phosphoinositide dependent protein kinase (PDK1) and Akt are thus recruited from the cytosol to the plasma membrane through their interaction with PIP3, and PDK1 then phosphorylates Akt at residues in the T-loop and/or C-terminal of the kinase domain. Phosphorylation at site Thr308 at T-loop plays major role in Akt activation, while phosphorylation at other sites promotes the interaction of AKT with other substrates and enhances their stability [260]. Activation of Akt occurs within minutes and, soon after activation, Akt dissociates from the plasma membrane and translocates to the nucleus [261]. Negative regulation of Akt activities occurs via two pathways: 1) active Akt inhibits the ability of tuberous sclerosis complex 2 (TSC2) by phosphorylating TSC2, which acts as a GTPase activating protein for the Akt activator Ras homolog enriched in brain (Rheb); 2) Akt

also activates mTORC1 by inhibiting the proline-rich Akt substrate of 40 kDa (PRAS40) via phosphorylation, thus blocking the inhibitory effect of PRAS40 on mTORC1. Once mTORC1 is activated, it phosphorylates IRS1, and this causes IRS1 degradation. In this way, Akt activation is aborted.

Akt signaling plays important roles in promoting cell proliferation/growth, inhibiting apoptosis, and an increasing metabolism [261]. Akt promotes survival of cells because it inhibits proteins that mediate apoptosis such as BAD. Akt phosphorylation of BAD promotes its binding to 14-3-3 proteins, and prevents its interaction with Bcl-X_L and thus suppresses apoptosis [262]. Akt also phosphorylates pro-apoptotic molecules such as the Fas ligand, which causes its sequestration in the cytoplasm, thus reducing its expression which interferes with apoptosis [263]. Studies have also shown that overexpression of Akt induces phosphorylation of apoptosis signal-regulated kinase 1 (Ask1), resulting in activation of JNK and p38 MAPK [264]. Activation of Akt leads to activation of eNOS, which promotes angiogenesis [265]. Akt also promotes cell proliferation by phosphorylating and activating CDK inhibitors such as p21 [266] and decreasing p27 levels (which is also a CDK inhibitor) [267]. Akt has also been shown to stimulate the translation of cyclin D proteins [268], and stabilize cyclin D1 proteins [269].

1.8.3 PPARs

PPARs belong to a subfamily of nuclear receptors that function as transcription factors. PPARs include three isoforms – PPARα, PPARγ and PPARβ/δ [270]. All three isoforms are expressed in endothelial cells including human aortic endothelial cells, carotid artery endothelial cells, and HUVEC [271, 272]. PPARs are comprised of a functional domain, a DNA binding domain and a ligand binding domain [273]. Upon ligand binding to PPAR, PPAR forms a heterodimer with a retinoid X receptor and this complex binds to specific peroxisome

proliferator response elements in the promoter regions of target genes. Then, following the conformation alternation of PPAR, a binding cleft is exposed for recruitment of transcription co-activators [270].

Fatty acids and their derivatives, oxylipins, are the natural ligands of PPARs, thus making PPARs important in lipid metabolism and homeostasis as well as other processes where lipids play a regulatory role [115]. PPARα can be activated by both SFAs and unsaturated fatty acids including palmitic acid, oleic acid, LA and AA [274], as well as some oxylipins such as 8-HETE [275]. PPARγ prefers PUFAs such as LA, ALA, AA and EPA as its ligands [276], as well as 9-HODE and 13-HODE produced from LA [277], and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [278]. SFAs and unsaturated fatty acids are also ligands of PPARδ [276]. Studies have shown that DGLA, EPA and AA have a weak affinity for PPARδ [279]. Generally, activation of both PPARα and PPARγ exert anti-inflammatory effects on endothelial cells because they inhibit ICAM-1, VCAM-2, interleukin (IL-6, IL-8), MCP-1, endothelin, and thrombin expression, and induce anti-angiogenic factors such as thrombospondin, while activation of PPARβ/δ exerts pro-angiogenic and pro-inflammatory effects on endothelial cells [280].

1.9 Concluding Statement

The literature indicates endothelial cells are critical mediators of vascular homeostasis, and they are highly responsive to an array of compounds that come from the diet. Among these compounds are fatty acids, which have been shown to modulate endothelial cell function, but how they operate is largely unknown. Therefore, to better understand this interaction, a series of experiments were performed *in vitro* to examine the relationship between fatty acids and their metabolites, specifically, oxylipins, in the activation of endothelial cells.

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Chapter 2: Rationale, Hypotheses and Objectives

2.1 Rationale

Endothelial cells line every blood vessel and therefore are present in all tissues and organs, and they play important roles in maintaining homeostasis within the circulatory system [1]. Healthy endothelial cells are normally confluent and in a quiescent and non-proliferative state, a condition in which they exhibit anti-inflammatory, anti-thrombotic, anti-coagulant, and anti-platelet aggregation properties. However, endothelial cells can be stimulated by signals such as angiogenic factors, shear stress, cytokines such as TNF-α and IL-1, and dietary factors such as PUFAs, which cause withdrawal from quiescence and enable entry into a state called endothelial activation. For example, activated endothelial cells can become proliferative, which allows them to participate in vessel repair, angiogenesis and plaque development. Activated endothelial cells resemble dysfunctional endothelial cells in that they exhibit increased permeability and greater adhesion molecule expression, and a switch to a pro-inflammatory and pro-thrombotic phenotype. The healthy endothelium is capable of returning to its quiescent state after the disappearance of activation factors, however, chronic endothelial activation is dangerous because these endothelial cells are not able to perform their normal functions and gradually enter a chronic inflammatory state termed endothelial dysfunction [2]. Endothelial dysfunction further enhances endothelial cell activation [3].

Endothelial dysfunction contributes to chronic inflammation within the vasculature, and is believed to be fundamental to many chronic diseases such as CVD and diabetes [4]. For example, dysfunctional endothelial cells produce less of the vasodilatory factors such as NO and PGI_2, which increases blood pressure that in turn further impairs endothelial cells. In the long

run, activated endothelial cells promote vessel remodeling and make the arteries become structurally stiff. As well, inflamed endothelial cells are responsible for the attraction and accumulation of immune cells, which further creates a pro-inflammatory environment and contributes to the buildup of 'foam cells' during development of atherosclerosis, and increases the risk of thrombosis due to the reduced secretion of anti-coagulant factors [5]. Endothelial cell dysfunction leads to a reduced production of NO, which is a key mediator of insulin-stimulated glucose uptake. This contributes to insulin resistance. Various metabolic symptoms associated with obesity/diabetes such as oxidative stress, hyperglycemia, and hyperlipidemia also contribute to endothelial dysfunction [6, 7].

Endothelial cell function plays a vital role in both the healthy and the diseased vasculature. Diet and nutrition are important factors in promoting health throughout the entire lifespan and in all age groups. Many dietary intervention studies have suggested that PUFAs are beneficial in promoting endothelial cell function. However, inconsistent results exist and some studies have reported adverse effects of both n-6 PUFA [8] and n-3 PUFAs [9]. Although experimental design, dose, and participant characteristics are factors that contribute to the variation of the results, the role of growth state (i.e. quiescent or proliferative) of endothelial cells has received limited attention. Testing this hypothesis *in vivo* is difficult, but *in vitro* systems allow investigation of individual PUFAs in a particular growth state while eliminating many other factors such as the interaction of PUFAs that are present in *in vivo* systems. Endothelial cell viability and proliferation are important characteristics because, on one hand, healthy endothelium requires cells to be viable and in a non-proliferative state, but vessel repair and angiogenesis requires cells to be in their proliferative state; on the other hand, the balance between endothelial cell viability and cell death determines whether endothelial cells are

contributing to normal development or disease progression [10-12]. While this knowledge gap is well recognized, comparing the effects of the individual major dietary PUFAs (LA, AA, ALA, EPA and DHA) in the same *in vitro* system on endothelial cell viability and proliferation in relation to growth state (quiescent, growing) has not been previously examined. Therefore, the current study was carried out to examine the role of growth state (quiescent, growing) in the EA.hy926 endothelial cell response to dietary n-6 PUFAs (LA, AA) and n-3 PUFAs (ALA, EPA, DHA). Furthermore, the MAPK pathway has been implicated in cell viability and proliferation [13], but its direct activation by PUFAs in both growing and confluent endothelial cells has not been investigated. Thus by using confluent cells to represent the healthy endothelium, and subconfluent cells to represent the injured endothelium, it was possible to examine the mechanism of action for each individual PUFA in the context of viability and proliferation .

Oxylipins are oxygenated PUFA derivatives that have been shown to participate in mediating endothelial cell function. However, due to the large numbers of different species and their complex synergistic or contradictory effects, their mechanism of action on endothelial cells is not understood. The final part of this thesis examined the role of oxylipins in modulating endothelial cell viability and proliferation by using inhibitors for oxylipin-producing enzymes. Due to the large number of CYP isoforms, preliminary studies focused on COX and LOX pathways. These data showed that cell viability and proliferation rate were changed to a greater degree by treatment with NDGA (5-LOX inhibitor) than with lornoxicam and suprofen (COX inhibitors) (Figure A.2). Studies in Chapter 3 showed that AA, EPA and DHA are potent in negatively affecting endothelial cell viability and proliferation compared to their precursor fatty acids (LA and ALA) (Figure 3.8). Preliminary studies also showed that combination treatment of DHA or AA with NDGA, but not lornoxicam or suprofen, further reduced cell viability and

proliferation compared to fatty acid treatment alone (Appendix Figure A.2). Hence, Chapter 5 investigated the effect of inhibition of 5-, 12- and 15-LOX pathways, on endothelial cell viability and proliferation, as well as the oxylipin profiles and potential molecular mechanisms associated with the changes.

2.2 Hypothesis and Objectives

The overall hypothesis of this research is:

Dietary PUFAs and oxylipins affect the viability and proliferation of EA.hy926 human endothelial cells through the differential activation of MAPKs in growing and confluent (quiescent) states.

This hypothesis was tested by dividing the overall hypothesis into three specific hypotheses:

Hypothesis #1: Substrates responsible for cell adherence (Matrigel, collagen, plastic surface) and growth states (growing, confluent) differentially affect EA.hy926 endothelial cell viability and proliferation (**Chapter 3**).

Objectives

- Investigate the effect of substrates (Matrigel, collagen, plastic surface) on EA.hy926 endothelial cell viability, proliferation and senescence as well as the potential molecular mechanisms by examining levels of proteins related to viability (caspase-3) and proliferation (MCM2, cyclin D1 and p27kip).
- Investigate the effect of PUFAs (LA, AA, ALA, EPA, DHA) on growing and confluent cell viability and proliferation when cells are grown on a Matrigel-coated surface or a plastic surface, as well as the potential molecular mechanisms by examining levels of proteins related to viability (caspase-3) and proliferation (MCM2, cyclin D1 and p27kip).

Hypothesis #2: DHA-induced apoptosis in EA.hy926 endothelial cells requires growth state (growing, confluent)-dependent activation of caspase-3 and MAPK (**Chapter 4**).

Objectives

- Investigate the type of cell death induced by DHA in confluent EA.hy926 cells.
- Investigate the molecular mechanisms of DHA-induced apoptosis in confluent cells in relation to caspase-3 and caspase-8, and to p38 MAPK.
- Investigate the effect of DHA on activation of MAPKs (p38 MAPK, ERK1/2, JNK) in growing and confluent cells.
- Investigate the role of MKK3/MKK6 and DUSP1 in p38 MAPK activation.

Hypothesis #3: 15-LOX inhibition, but not 5- or 12-LOX inhibition, negatively affects EA.hy926 endothelial cell viability and proliferation in a growth state (growing, confluent)-dependent manner (**Chapter 5**).

Objectives

- Investigate whether inhibition of LOX pathways (5-, 12-, 15-LOX) affects the viability and proliferation of growing and confluent EA.hy926 endothelial cells.
- Investigate the molecular mechanisms of 15-LOX inhibition on cell viability and proliferation in relation to p38 MAPK, Akt and PPARα as well as potential cross-talk in signaling among these molecules in growing and confluent endothelial cells.
- Investigate the oxylipin profile associated with the 15-LOX inhibition in growing cells.

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Chapter 3: Importance of Extracellular Matrix and Growth State for the EA.hy926 Endothelial Cell Response to Polyunsaturated Fatty Acids

3.1 Contribution Statement

I, Youjia Du, made the following contribution to this study:

- Developed the study concept and experimental design with other contributors
- Conducted all experiments and data analysis
- Wrote the manuscript
- Interpreted results and edited the manuscript with other contributors

Other contributors:

- Peter Zahradka
- Carla G. Taylor
- Harold M. Aukema

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3.2 Study Rationale

The EA.hy926 endothelial cell line has been used to study various properties of endothelial cells over the decades since it was created in 1983 [1], and has shown to be a robust cell line that possesses the features of endothelial cells such as vWF and E-selectin [2]. Endothelial cells in blood vessels rest on a layer of extracellular matrix, which not only provides structural support to the cells, but also influences cell function. The proteins that compose the ECM, such as collagen, fibronectin, and laminin, are widely used as substrates for the culture of endothelial cells. Matrigel, a composite mixture of ECM proteins, has been used to study angiogenesis/tube formation by endothelial cells, but it has not been used as a substrate for growing endothelial cells. So far, characterizing the growth of EA.hy926 endothelial cells and selecting the most suitable substrates for cell growth has received limited attention. Dietary PUFAs, components of the lipid molecule family, are better known for their ability to produce eicosanoids, which play important roles in inflammation and in cell signaling. Although many dietary intervention studies and *in vitro* studies have demonstrated that PUFAs can influence endothelial function, their mechanisms of action are still not fully understood. A simultaneous comparison of the effects of major PUFAs (LA, AA, ALA, EPA and DHA) on the viability and proliferation of endothelial cells has not been completed. At same time, whether growth state plays a role in the endothelial cell response to PUFAs has not been investigated. Therefore, this project begins with characterizing the growth of EA.hy926 endothelial cells on different substrates (Matrigel, collagen and plastic surface), and studies the viability and proliferation of the cells in response to various concentrations of PUFAs (LA, AA, ALA, EPA and DHA) when they are grown on different substrates and at different growth states (growing vs confluent).

3.3 Abstract

Consumption of different PUFAs can induce functional changes in blood vessels via endothelial cells, which interact with dietary factors in the circulation. The basement membrane that separates the endothelium from the smooth muscle cells of the medial layer can also influence the functional state of endothelial cells. However, the effect of basement membrane on the endothelial response to dietary PUFAs in relation to growth state (e.g. proliferation versus quiescence) has not been reported. We therefore compared the viability (CCK kit) and proliferation (bromodeoxyuridine incorporation) of EA.hy926 endothelial cells grown on Matrigel or collagen versus non-coated plates. EA.hy926 viability and proliferation were also assessed after treatment with 0-150 μM of PUFAs (LA, AA, ALA, EPA and DHA). Our study showed that only cells grown on Matrigel-coated plates reached quiescence after becoming confluent with a decreased level of MCM2 and p-cyclin D1 (T286), increased levels of p27kip1 and a low level of apoptosis and senescence. AA, EPA and DHA decreased the viability and proliferation of subconfluent cells grown on plastic dishes in a dose-dependent manner, while the presence of Matrigel made the cells resistant to these adverse effects. Confluent cell viability was less sensitive to higher concentrations of AA, EPA and DHA than subconfluent cells, and a significant increase in caspase-3 cleavage was only observed in confluent cells treated with DHA. Higher concentrations of AA, EPA and DHA suppressed DNA synthesis by both subconfluent and confluent cells, while precursor C18 PUFAs (LA and ALA) had no negative effects on viability and proliferation. Our study is the first to show that extracellular matrix and growth state are important factors in the EA.hy926 cell response to PUFAs, and that the mechanisms by which individual PUFAs operate may be growth state-dependent.

3.4 Introduction

The endothelium is formed by a single layer of endothelial cells and lines the luminal surface of all blood vessels. Healthy endothelial cells play an important role in maintaining vascular homeostasis [3], and function as a barrier that is selectively permeable for the transportation of key molecules. Endothelial cell proliferation and migration also contribute to the formation of blood vessels in response to the influence of various hormones. Endothelial cells regulate vascular tone by secreting vasodilators such as NO and $PGI_{2,}$ and vasoconstrictors such as endothelin. Endothelial cells are responsible for creating the anti-thrombotic and anti-coagulant environment required for the circulation. Moreover, endothelial cells participate in inflammatory responses by expressing adhesion molecules, as well as metalloproteinases. Endothelial cells are attached to the basement membrane, an ECM protein-rich layer located beneath the endothelium that provides physical and chemical support. Adhesion of endothelial cells to basement membrane proteins through integrins is important for maintaining endothelial cell viability and strongly influences other functions such as proliferation, migration, vessel formation and blood vessel stabilization [4].

In plasma, total NEFA levels can reach 300 μM in healthy individuals and higher in persons with type 2 diabetes [5]. At these concentrations, FAs could have a major influence on vascular physiology. Based on dietary intervention studies that examined how modulation of endothelial function by different dietary PUFAs affects vascular function, it is believed that incorporating n-3 PUFA into the diet improves endothelial function as indicated by flow-mediated dilatation [6-9]. However, the mechanism of action for PUFA, particularly in relation to specific endothelial processes, has received limited attention.

The functional properties of endothelial cells are strongly influenced by the substrate on which they grow [10-12]. Dishes coated with ECM proteins such as collagen, gelatin and laminin

are used for culturing adherent cells such as endothelial cells. Matrigel, which is composed of a mixture of ECM proteins derived from tumor cells, is an alternative substrate. Interestingly, little attention has been paid to how ECM influences the viability and proliferation of endothelial cells, which are critical factors contributing to the role of endothelial cells in normal vascular physiology as well as pathophysiology. In the healthy endothelium, endothelial cells are confluent and non-proliferating. In contrast, endothelial cells must proliferate when the endothelium has been damaged and is under repair. It is recognized that certain dietary factors can damage the endothelium, but interestingly, fats are associated with both positive and negative effects on the endothelium. However, the effect of PUFAs on the viability and proliferation of endothelial cells in the context of both ECM substrate and growth state has not been investigated.

To address these concepts, the current study investigated how EA.hy926 endothelial cells, a fusion of human umbilical vein endothelial cells with human carcinoma A549 cells [1], respond to different n-6 (LA and AA) and n-3 (ALA, EPA and DHA) PUFAs in terms of viability and proliferation when they are grown on different ECM substrates. The response of EA.hy926 endothelial cells to PUFAs in different growth states (i.e., growing and confluent) was considered in relation to proteins involved in cell viability and proliferation as a means of investigating their mechanisms of action.

3.5 Materials and Methods

Cell culture

EA.hy926 endothelial cells were purchased from ATCC (cat. CRL 2922, Manassas, Virginia, USA) and propagated according to the manufacturer's protocol. EA.hy926 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 20 mM HEPES, 100 units/mL penicillin, and 100 µg/mL streptomycin supplemented with 10% fetal bovine serum (FBS). The

cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Cells were used within 20 passages.

ECM coatings

Growth Factor Reduced Matrigel (Corning®, Product No 356231) was handled according to the manufacturer's protocol. Each well of the 96-well plate received 100 μL of DMEM containing 5 μL of Matrigel. The plates were left for 1 hour at room temperature and were washed once with DMEM before seeding the cells. Collagen Type I (Sigma, Product No. C7661) was dissolved in acetic acid according to the manufacturer's protocol, and concentrations of 6 and 10 $\mu g/cm^2$ (manufacturer's suggested lower and upper concentrations) were used to coat the dishes for 5 hours at 37°C after which the liquid was removed and the well allowed to dry overnight under a UV light. The coated surface was rinsed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) before cell seeding.

Cell preparation

EA.hy926 endothelial cells were seeded at a density of 9000 cells/cm^2. The day cells were seeded was referred to as day 0. Subconfluent cells were used at day 4, and confluent cells were used at day 8. EA.hy926 endothelial cells were grown on Matrigel or collagen or non-coated plates for up to 12 days.

PUFA treatment

Polyunsaturated fatty acids (LA, AA, ALA, EPA and DHA; Cayman Chemical) were dissolved in ethanol and then mixed with 5% fatty acid free bovine serum albumin in PBS. They were added directly to the cell culture medium without disturbing the cells. Vehicle control consisted of ethanol, fatty acid free bovine serum albumin and PBS.

Cell viability

Cell viability (total cell number) was assessed with the CCK-8 (cell counting kit, Dojindo Molec Tech) assay. CCK reagent (water-soluble tetrazolium salt) was added to the cells and incubated for 90 min. It was reduced by mitochondria dehydrogenase to water-soluble yellow-coloured formazan. The amount of formazan generated is directly proportional to the number of living cells, which can be detected by reading the absorbance of the media measured at 450 nm using a microplate reader.

Cell proliferation

Proliferation of cells was measured with the Bromodeoxyuridine (BrdU) Cell Proliferation kit (Millipore). BrdU reagent was added to the cells and incubated for 4 h. Cells were fixed, DNA was denatured, and incorporated BrdU was detected immunochemically according to the kit instructions. Plates were measured at a dual wavelength of 450/550 nm.

Western blotting

Protein was extracted from EA.hy926 cells grown on Matrigel-coated and plastic culture plates. Cells were lysed each day (day 4 to day 12) with 2× sample buffer (125 mM Tris-HCl pH 6.8, 2% Sodium dodecyl sulphate, 20% glycerol) and briefly sonicated. Subconfluent and confluent EA.hy926 cells grown on Matrigel-coated plates were also lysed and sonicated after treatment with 125 μM of PUFAs (LA, AA, ALA, EPA and DHA) for 8 h. Twenty μg of lysate protein were subjected to SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes for immunoblotting. Primary antibodies were diluted in TBST (Tris-buffered saline with Tween-20; 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.05% Tween 20) containing 3% bovine serum albumin (BSA). The

primary antibodies employed were rabbit anti-MCM2 (1:1000, abcam, #4007), rabbit anti-phospho-cyclin D1 (Thr286) (1:1000, Cell Signaling, #3300), mouse anti-cyclin D1 (1:1000, Cell Signaling, #2926), rabbit anti-VE (vascular endothelial)-cadherin (1:1000, Cell Signaling, #2500), rabbit-anti-p27 kip1 (1:1000, Cell Signaling, #3686), rabbit anti-cleaved caspase-3 (1:1000, Cell Signaling, #9664) and rabbit anti-caspase-3 (1:1000, Cell Signaling, #9662). Horseradish peroxidase (HRP) conjugated secondary antibodies were used at a dilution of 1:10000 in TBST containing 1% BSA. The relative band intensities were captured using a FluorChem® Q imager and quantified with AlphaView software (Alpha Innotech). The Western blot data were normalized to a Ponceau S stained image of the blot.

Senescence β-Galactosidase staining

Senescence was detected using the Senescence β-Galactosidase Staining Kit (Cell Signaling, #9860). EA.hy926 cells were rinsed with PBS, then fixed and stained with X-Gal according to the manufacturer's protocol. Cells were incubated in a 37°C incubator (no CO_2) overnight and imaged under a light microscope (200× magnification). X Gal was stained blue when cleaved by β-galactosidase.

Statistical Analysis

One-way ANOVA and Tukey's test were used to assess statistical significance, which was set at $p<0.05$ (SPSS version 20). All experiments were independently repeated at least 3 times. Within each experiment, the plotted values were derived from treatments done in triplicate (n=3) and are expressed as mean ± SEM.

3.6 Results

Effects of basement membrane

Growth of endothelial cells with Matrigel and without (control - plastic surface) had no significant effect on viability or total cell number (Figure 3.1A). Cells reached a maximal number at day 8 and this was defined as confluence. Similarly, no significant differences in cell viability or total cell number were observed when cells were grown on collagen-coated plates, at two different concentrations of collagen (Figure 3.1B). The cells grown without Matrigel (Panel A) served as the no collagen control (Panel B) for this experiment. Visual confirmation that cells grown on both Matrigel and plastic reached confluence on day 8 is shown in Figure 3.1C and Figure 3.1D, respectively.

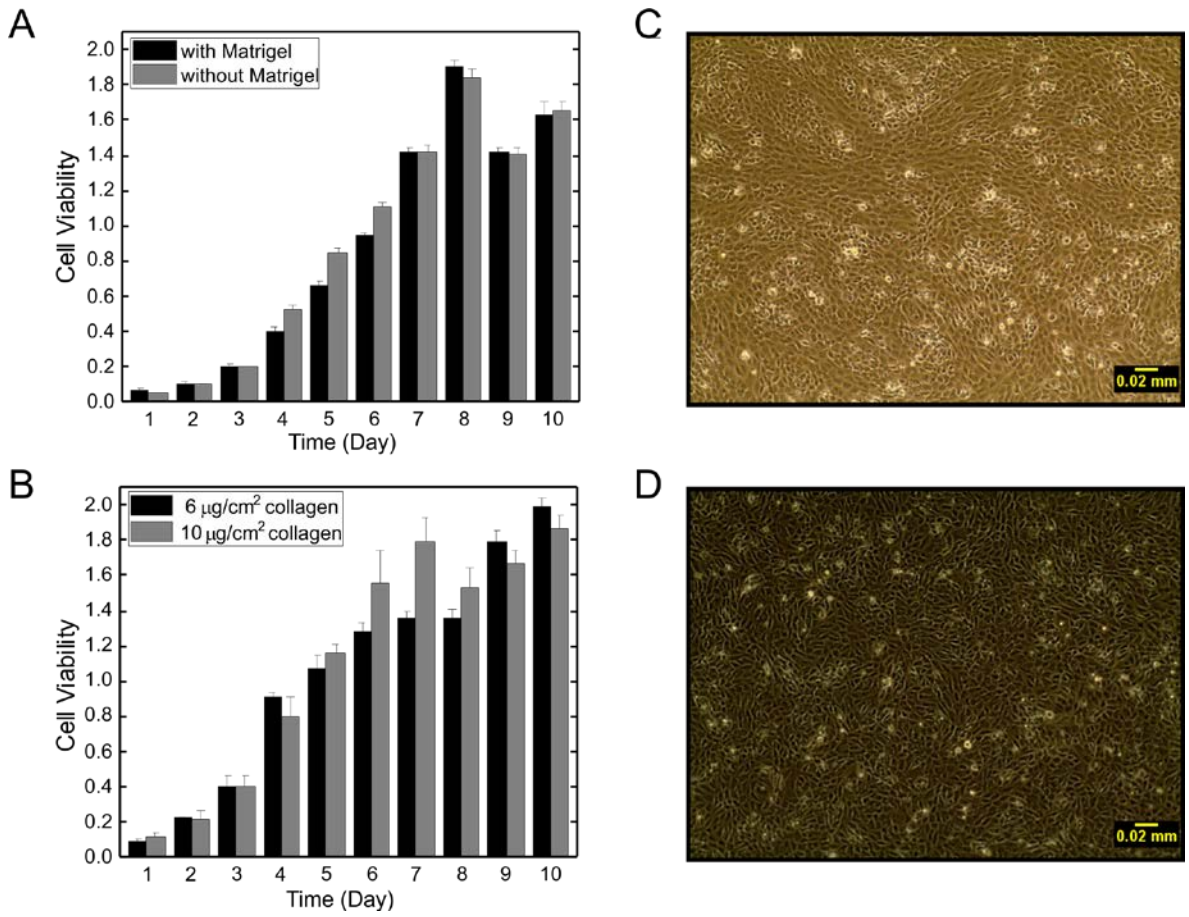


Figure 3.1 Effect of ECM substrate on growth of EA.hy926 cells.

EA.hy926 endothelial cells were seeded at 9000 cells/cm^2 on 96 well plates pre-coated with Matrigel (5 μL Matrigel mixed in 95 μL DMEM) or without Matrigel (Panel A) or Collagen Type I dissolved in acetic acid at concentrations of 6 and 10 μg/cm^2 (Panel B) and grown for up to 10 days without changing the media. Cell number was determined with the CCK-8 assay. Cells grown with Matrigel (Panel C) and without Matrigel (Panel D) were imaged at day 8 with a light microscope at 100×. A microplate reader was used to read absorbance of the media at 450 nm. Data are plotted as means ± SEM (n=3).

When EA.hy926 endothelial cells were grown on non-coated plates, the DNA synthesis rate peaked on day 5, but DNA synthesis remained elevated to day 10 (Figure 3.2A) even though the cells visually reached confluence at day 8. When EA.hy926 endothelial cells were grown on Matrigel, the DNA synthesis rate reached a maximum on day 4 and then started to decrease after day 8 (Figure 3.2B), suggesting that the cells became quiescent after reaching confluence (day 8) on Matrigel-coated plates. However, when EA.hy926 endothelial cells were grown on collagen coated plates, the DNA synthesis rate remained high from day 4 to day 10 (Figure 3.2C), similar to cells grown without basement membrane. Thus, subsequent experiments focused on cells grown on plastic plates with and without Matrigel as the substrate.

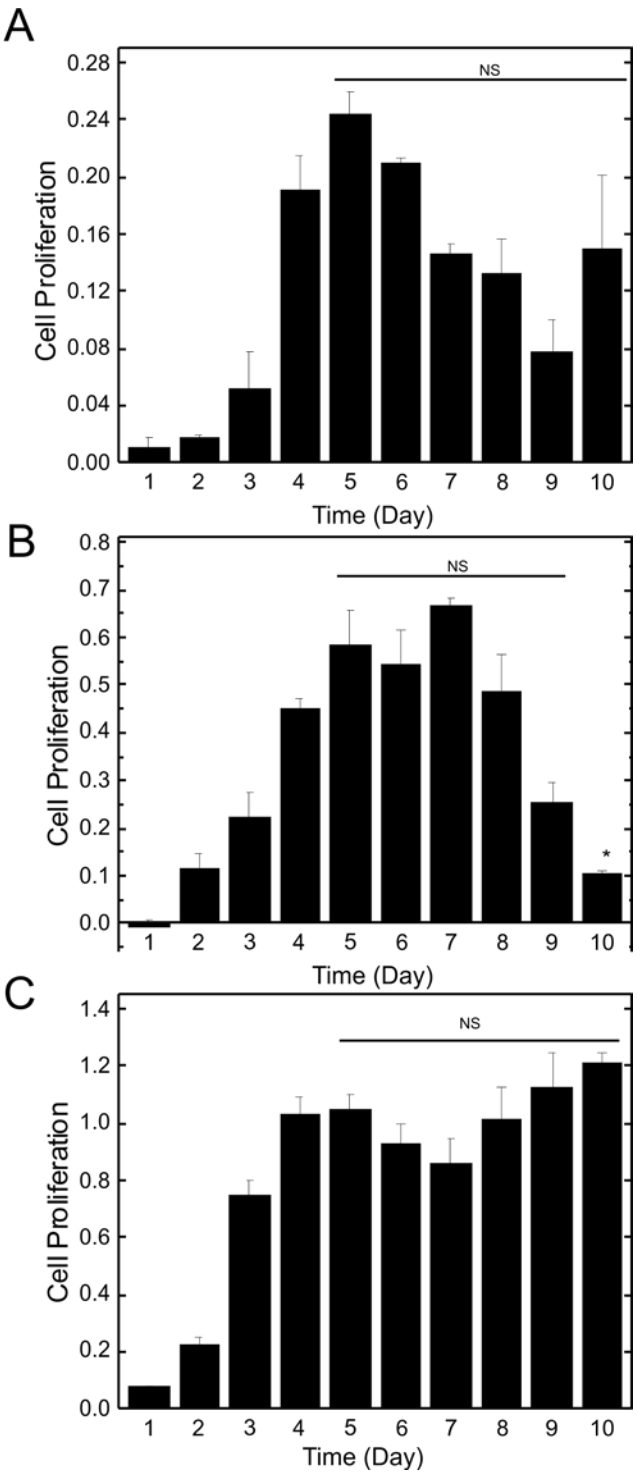

A
Cell Proliferation
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
NS
1 2 3 4 5 6 7 8 9 10
Time (Day)
B
Cell Proliferation
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NS
*
1 2 3 4 5 6 7 8 9 10
Time (Day)
C
Cell Proliferation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NS
1 2 3 4 5 6 7 8 9 10
Time (Day)

Figure 3.2 Effect of ECM substrate on proliferation of EA.hy926 cells.

EA.hy926 endothelial cells were seeded at a density of 9000 cells/cm^2 on non-coated (Panel A), Matrigel-coated (Panel B) or collagen-coated (Panel C) 96 well plates and grown for up to 10 days without changing the media. Cell proliferation was assessed with the BrdU cell proliferation assay. Plates were read at dual wavelength 450/550 nm. Data are plotted as means ± SEM (n=3). NS, non-significant versus day 4; *significantly different ($p < 0.05$) from day 4.

To verify the DNA synthesis measurements, Western blotting was used to examine expression of MCM2, p-cyclin D1 (T286), cyclin D1 and VE-cadherin in cells grown on Matrigel-coated and plastic plates. MCM2 is a key component of the MCM2-7 complex that is essential for DNA replication initiation and elongation in eukaryotic cells. MCM2 peaked at day 5 in cells grown on Matrigel-coated plates and started to decline afterwards, and this suggested the cells were entering a quiescent state after reaching confluence (Figure 3.3B). This interpretation was tested with p-cyclin D1, an important regulator in cell cycle progression that contributes to the entry from G1 to S phase. Since phosphorylation at T286 facilitates cyclin D1 nuclear export to the cytoplasm for degradation and thus progression through the cell cycle, a decline of the p-cyclin D1 (T286)/cyclin D1 ratio (Figure 3.3C) in the confluent cells was considered an indication of a decrease in actively cycling cells. VE-cadherin, which is endothelial specific and belongs to the adherens junction family, peaked on day 7 (Figure 3.3D). The presence of VE-cadherin indicated intercellular junctions had formed, thus confirming the EA.hy926 cells were a confluent monolayer. In contrast, when cells were grown on plastic plates, the p-cyclin D1 (T286)/cyclin D1 ratio remained high until day 12 when it reached its greatest elevation (Figure 3.4C). These results indicate that the cells were not entering a quiescent state even if MCM2 levels had declined (Figure 3.4B). The VE-cadherin level was also high (Figure 3.4D) suggesting that cell-cell contacts were continuously present, and this is supported by evidence of barrier formation through the development of electrical resistance as indicated by TEER (TransEpithelial Electrical Resistance) which was measured using Millicell ERS-2 Electrical Resistance System (data not shown).

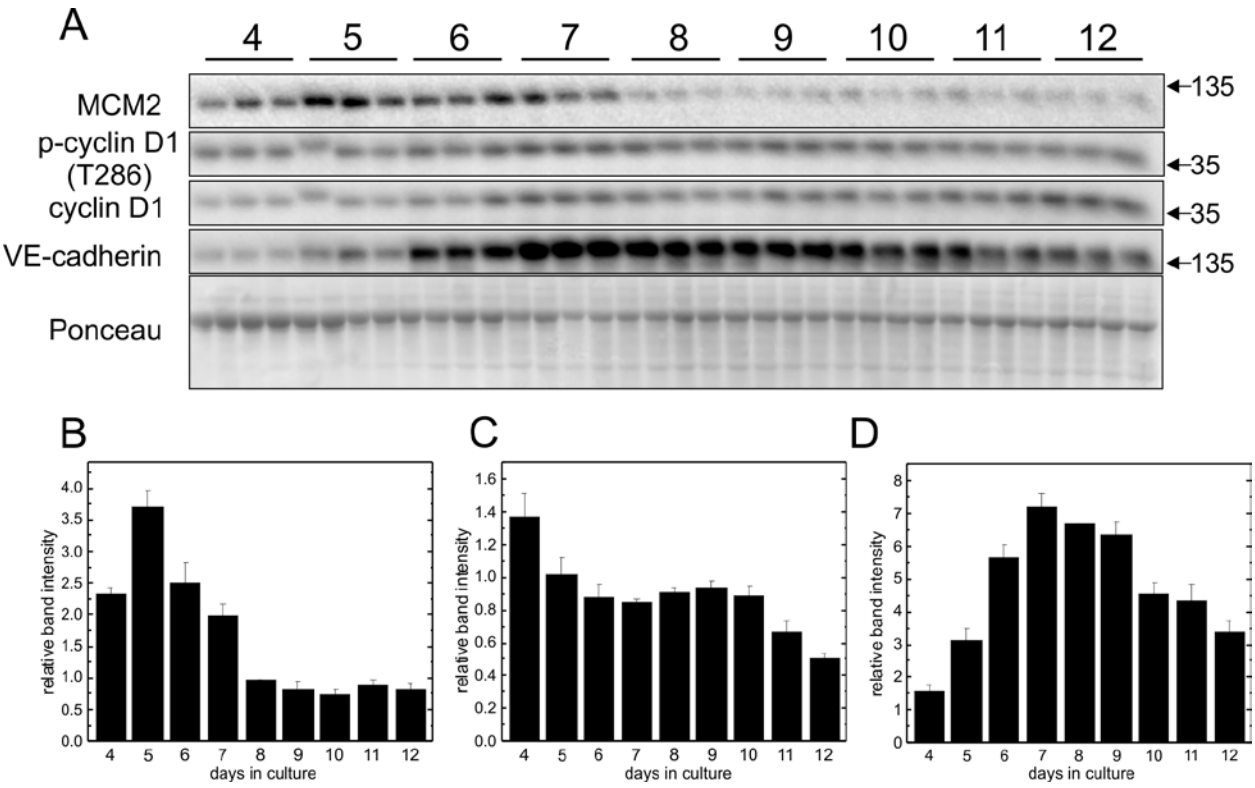


Figure 3.3 Time course for MCM2, p-cyclin D1, cyclin D1 and VE-cadherin in EA.hy926 cells grown on Matrigel-coated plates.

EA.hy926 endothelial cells were seeded on Matrigel-coated plates at 9000 cells/cm^2 and grown for 12 days. Protein levels of MCM2, p-cyclinD1, cyclin D1 and VE-cadherin were determined by Western blotting of cell lysates prepared each day from day 4 to day 12. Representative blots showing 3 lanes per treatment are presented in Panel A. Densitometry was used to quantify the intensity of the bands in the upper panel and data were normalized to a band visualized by

Ponceau staining. Data are presented as means ± SEM (n=3) for MCM2 (Panel B), p-cyclin D1/cyclin D1 (Panel C) and VE-cadherin (Panel D).

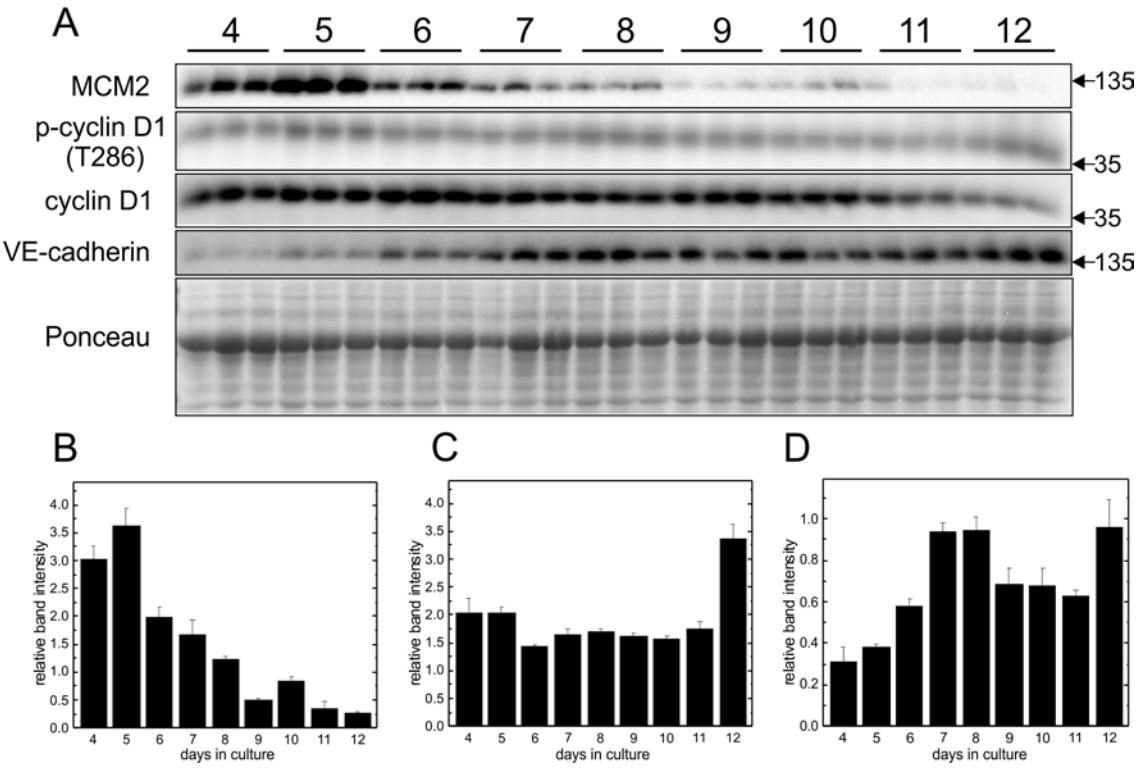


Figure 3.4 Time course for MCM2, p-cyclin D1, cyclin D1 and VE-cadherin in EA.hy926 cells grown on plastic culture plates.

EA.hy926 endothelial cells were seeded on plastic plates at 9000 cells/cm^2 and grown for 12 days. Protein levels of MCM2, p-cyclinD1, cyclin D1 and VE-cadherin were determined by Western blotting of cell lysates prepared each day from day 4 to day 12. Representative blots showing 3 lanes per treatment are presented in Panel A. Densitometry was used to quantify the intensity of the bands in the upper panel and data were normalized to a band visualized by Ponceau staining. Data are presented as means ± SEM (n=3) for MCM2 (Panel B), p-cyclin D1/cyclin D1 (Panel C) and VE-cadherin (Panel D).

To confirm the conclusion that cells grown on Matrigel enter a quiescent state after reaching confluence, expression of p27kip1, cleaved caspase-3 and caspase-3, and β-galactosidase activity were examined in cells grown on Matrigel-coated or plastic plates. p27kip1 is an important negative regulator of CDK and limits cell cycle progression from G1 to S phase. The higher levels of p27kip1 in cells grown on Matrigel-coated plates after day 4 (Figure 3.5A) compared to cells grown on plastic plates (Figure 3.5B) demonstrated that cell cycle progression has been greatly limited in cells with Matrigel as the substrate (Figure 3.5A). Caspase-3 activation is a key mediator and hallmark of apoptosis. The ratio of cleaved caspase-3/caspase-3 peaked on day 10 and continued at high levels in cells grown on plastic plates (Figure 3.6B), while the ratio in cells grown on Matrigel (Figure 3.6A) was relatively lower. Senescence-associated (SA) β-galactosidase activity, which is specifically detected at pH 6.0, is a widely used biomarker of cell senescence [13]. Figure 3.7 shows that there were more senescent cells present in cultures grown on plastic plates (Figure 3.7B) than in cultures grown on Matrigel-coated plates (Figure 3.7A). This finding indicates that cells grown on plastic have greater potential to become senescent, while cells grown on Matrigel-coated plates are less likely to be senescent. Therefore, confluency appears to promote entry of cells into a quiescent stage characterized by low levels of DNA synthesis and SA-β-galactosidase.

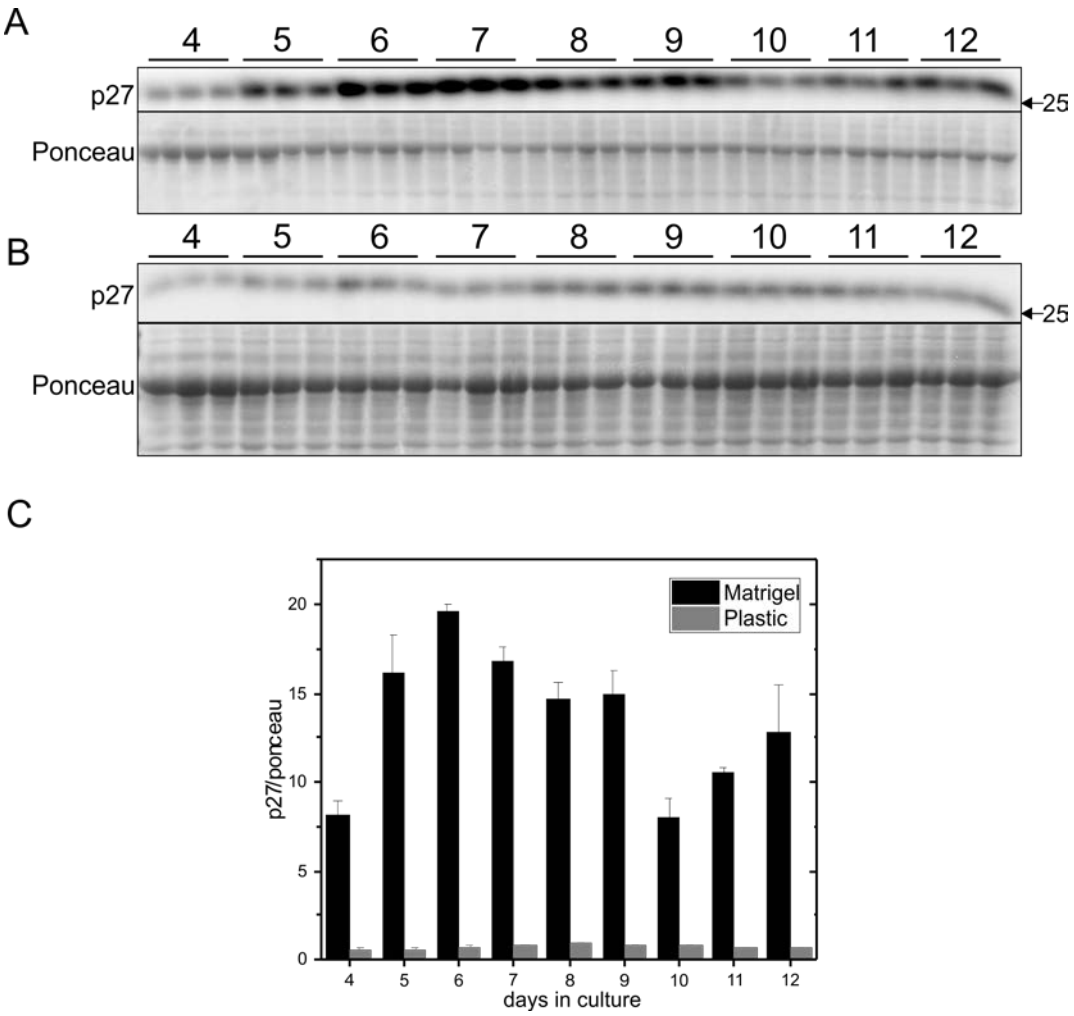


Figure 3.5 Time course of p27kip1 in EA.hy926 cells grown on Matrigel-coated or plastic culture plates.

EA.hy926 endothelial cells were seeded on plastic plates at 9000 cells/cm^2 and grown for 12 days. Protein levels of p27kip1 were determined by Western blotting of cell lysates prepared each day from day 4 to day 12. Representative blots showing 3 lanes per treatment are presented in Panel A (Matrigel) and B (Plastic). Densitometry was used to quantify the intensity of the bands in Panel A and B, and data were normalized to a band visualized by Ponceau staining (Panel C). Data are presented as means ± SEM (n=3).

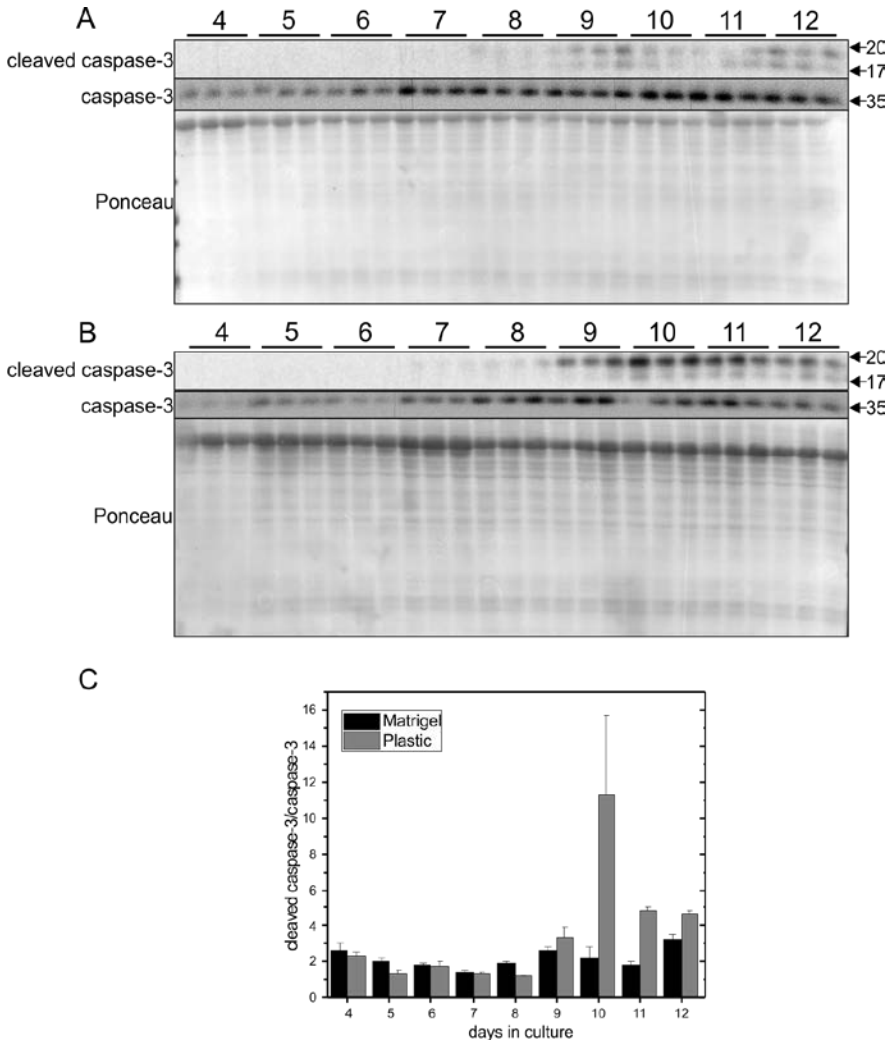


Figure 3.6 Time course of cleaved caspase-3 and caspase-3 in EA.hy926 cells grown on Matrigel-coated or plastic culture plates.

EA.hy926 endothelial cells were seeded on plastic plates at 9000 cells/cm^2 and grown for 12 days. Protein levels of cleaved caspase-3 and caspase-3 were determined by Western blotting of cell lysates prepared each day from day 4 to day 12. Representative blots showing 3 lanes per treatment are presented in Panel A (Matrigel) and B (Plastic). Densitometry was used to quantify the intensity of the bands in Panel A and B, and data were normalized to a band visualized by Ponceau staining (Panel C). Data are presented as means ± SEM (n=3).

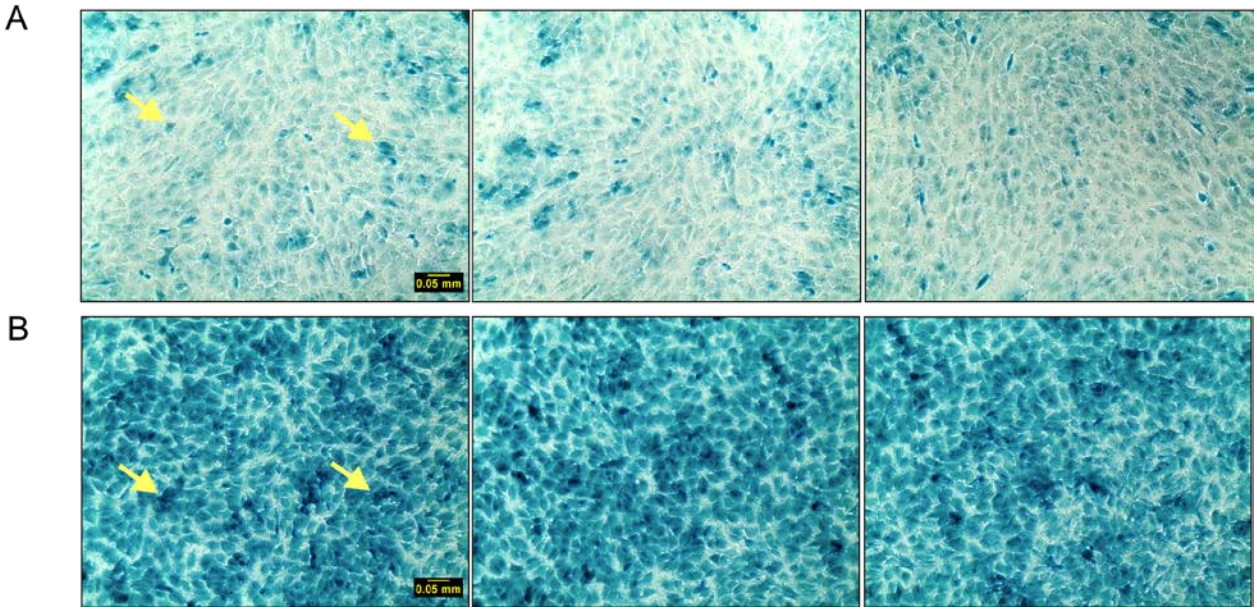


Figure 3.7 Senescence-associated β-Galactosidase Staining in EA.hy926 cells grown on Matrigel-coated or plastic culture plates.

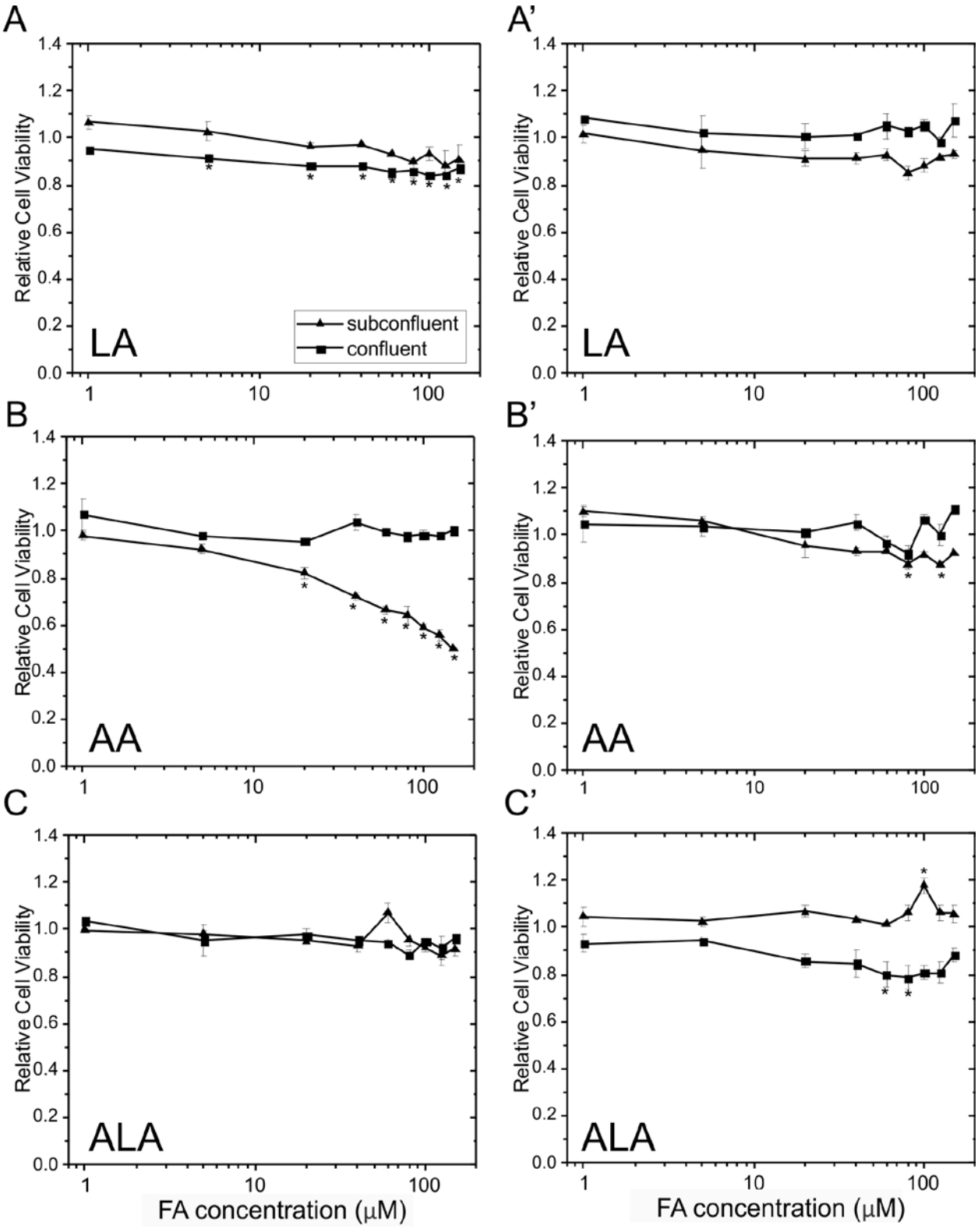
EA.hy926 endothelial cells were seeded on Matrigel-coated (Panel A) or plastic plates (Panel B) at 9000 cells/cm^2 and grown for 10 days. SA-β-Galactosidase Staining was performed on cells and 3 representative images are shown per condition. The arrows in the first image of Panel A and Panel B indicate cells with a high level of SA-β-galactosidase staining.

Effects of PUFAs

PUFAs (final concentrations of 1, 5, 20, 40, 60, 80, 100, 125, and 150 μM) were added on day 4 to subconfluent and on day 8 to confluent cells grown without (Figure 3.8A-E and Figure 3.9A-E) and with Matrigel (Figure 3.8A'-8E' and Figure 3.9A'-E'). The cells were then incubated for 24 hours with the PUFA treatment, and viability (Figure 3.8) was determined with the CCK-8 kit. AA (Figure 3.8B), EPA (Figure 3.8D) and DHA (Figure 3.8E) decreased the viability of subconfluent cells grown without Matrigel in a dose-dependent manner, while LA (Figure 3.8A) and ALA (Figure 3.8C) had no negative effect. Compared to vehicle control, AA significantly decreased the viability of proliferating cells at 20 μM, while EPA and DHA began to reduce viability at 5 μM and 60 μM, respectively. In contrast, the viability of subconfluent cells when grown on Matrigel was unaffected by any of the PUFA treatments (Figure 3.8A'-E') except for DHA which reduced cell viability by about 20% at 125 and 150 μM. Although there were significant differences in viability observed at certain individual concentrations (ALA at 100 μM, AA at 80 and 125 μM in the depicted experiment for subconfluent cells), these effects were not consistently seen in replicate experiments.

The viability of confluent cells grown without Matrigel was generally unaffected by treatment with different PUFA except for LA (Figure 3.8A) and DHA (Figure 3.8E). Although viability was significantly decreased by LA at concentrations higher than 5 μM, the effect was small (-13%). DHA at 20 and 40 μM significantly increased the viability of confluent cells in the experiment shown, however, this was not observed in the replicate experiments. Viability of confluent cells grown on Matrigel was unaffected by treating the cells with different PUFAs except for ALA (Figure 3.8C') and DHA (Figure 3.8E'). However, the differences detected for ALA at 60 and 80 μM were of small magnitude and not observed at higher concentrations or in

replicate experiments. In contrast, DHA dose-dependently reduced cell viability by 12% and 30% at 125 μM and 150 μM, respectively, for confluent cells grown on Matrigel.


A
A'
B
B'
C
C'
Relative Cell Viability
subconfluent
confluent
LA
AA
ALA
FA concentration (μM)

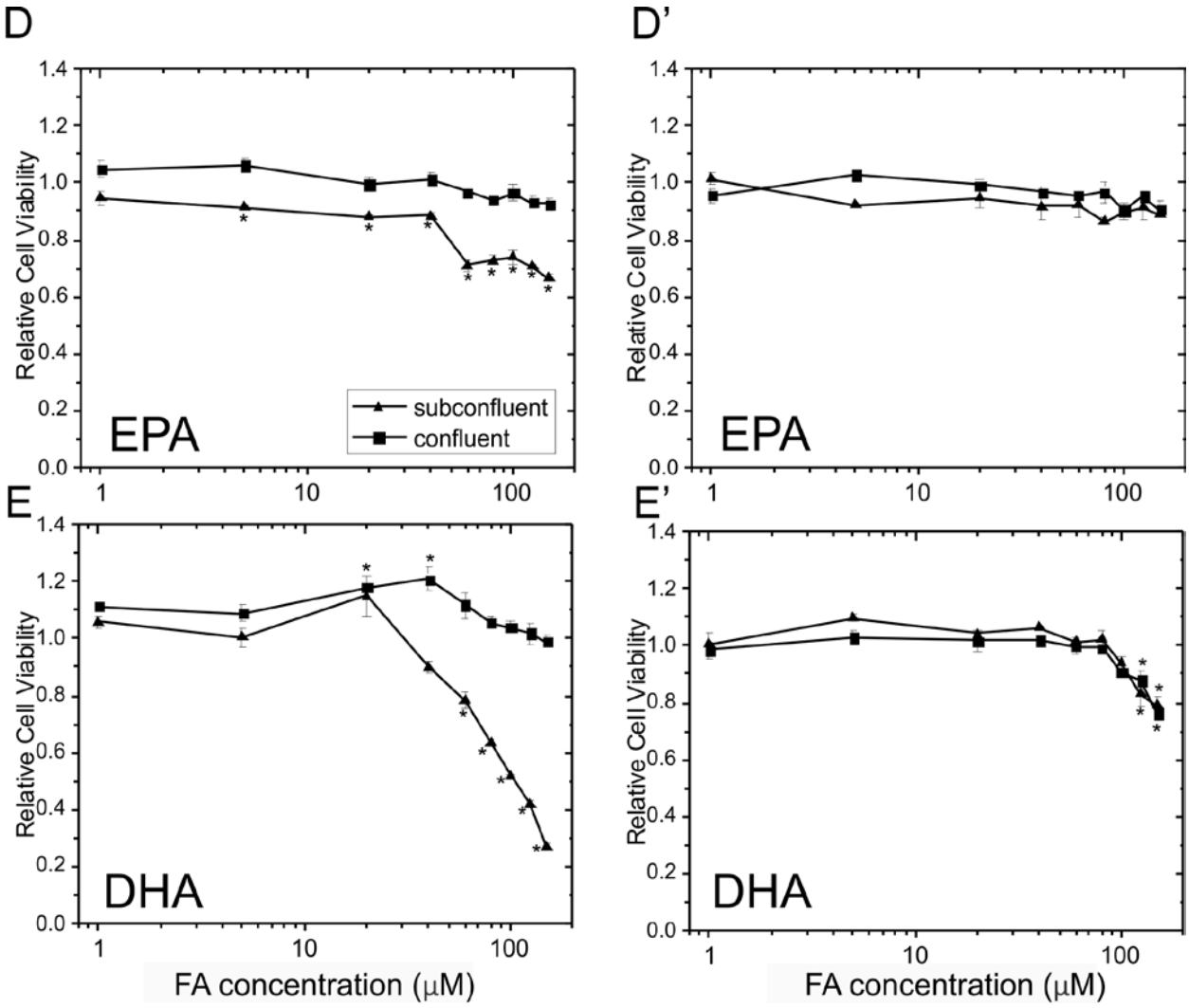
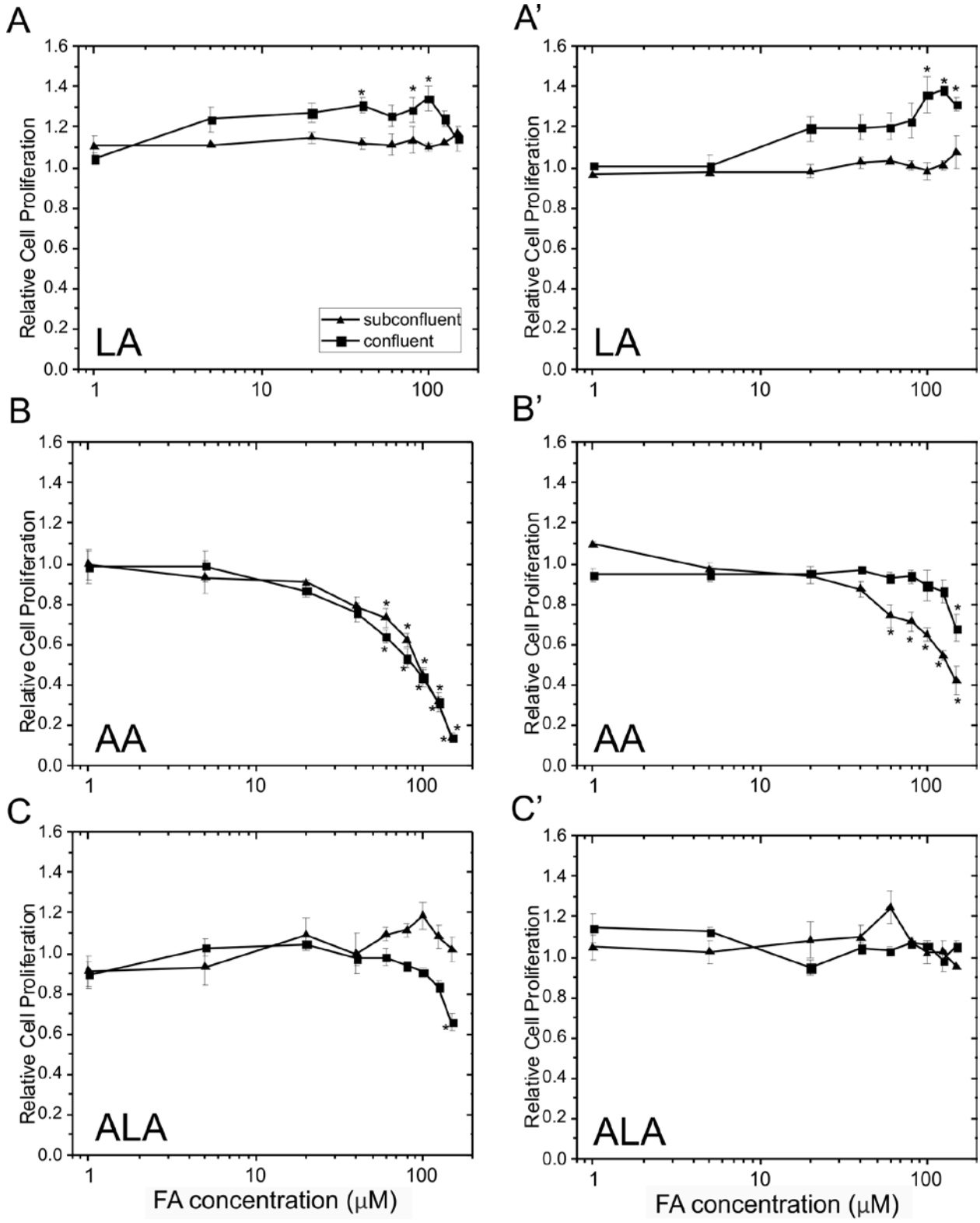


Figure 3.8 Effect of ECM substrate and growth state on viability of EA.hy926 cells in presence of PUFAs.

EA.hy926 endothelial cells were seeded on plastic culture plates at 9000 cells/cm^2 in the absence (Panel A-E) or presence of Matrigel (Panel A'-E'). Growing cells (day 4) or confluent cells (day 8) were treated with different PUFAs (LA [Panel A, A'], AA [Panel B, B'], ALA [Panel C, C'], EPA [Panel D, D'] and DHA [Panel E, E']) at final concentrations of 1, 5, 20, 40, 60, 80, 100, 125 and 150 μM for 24 hours, at which point, cell viability was assessed using the CCK-8 assay. Data are normalized with vehicle control values and plotted as means ± SEM (n=3).

*Significantly different ($p < 0.05$) from the vehicle control for the respective growth state.


A
A'
B
B'
C
C'
Relative Cell Proliferation
LA
AA
ALA
subconfluent
confluent
FA concentration (μM)
1
10
100
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

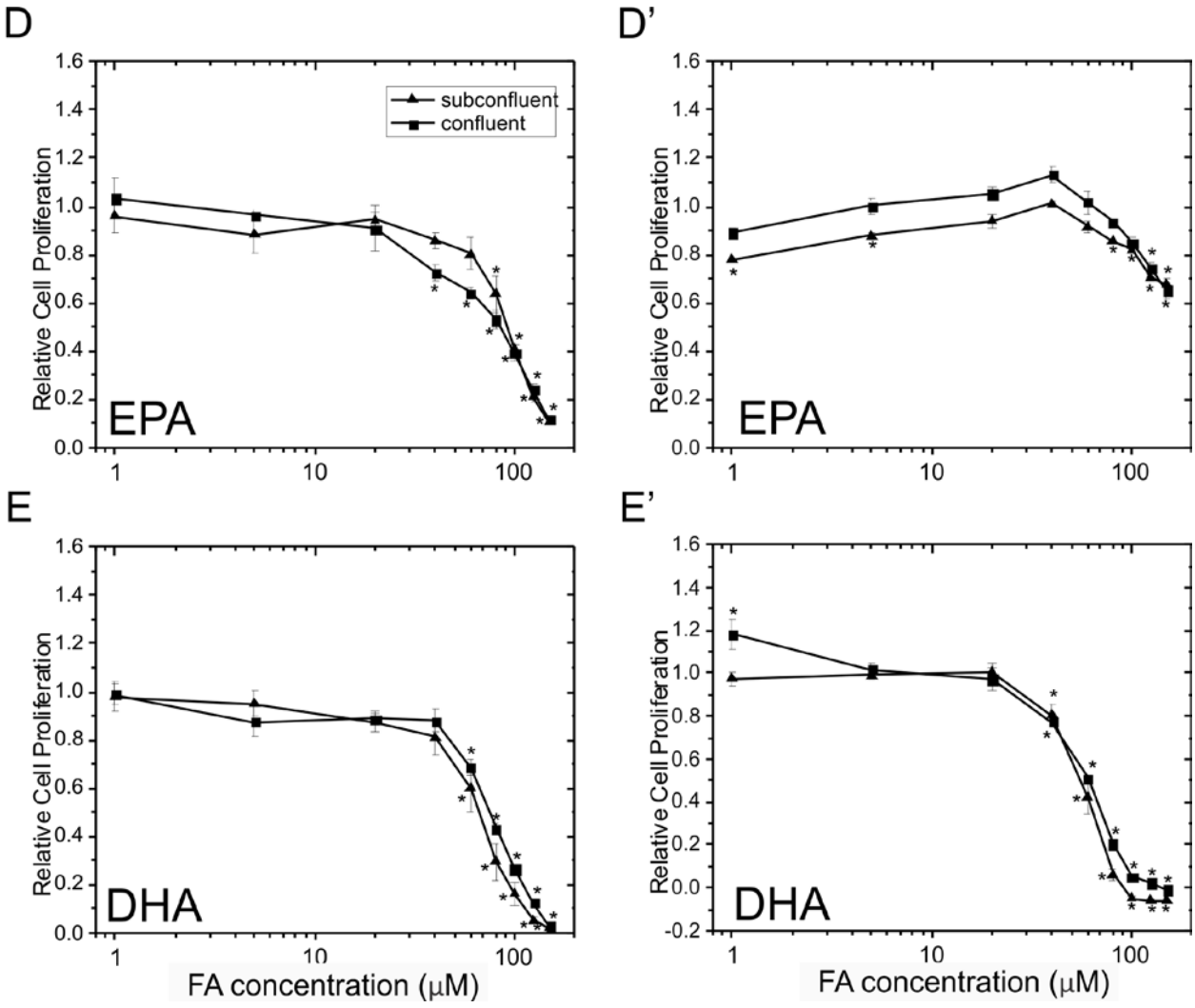
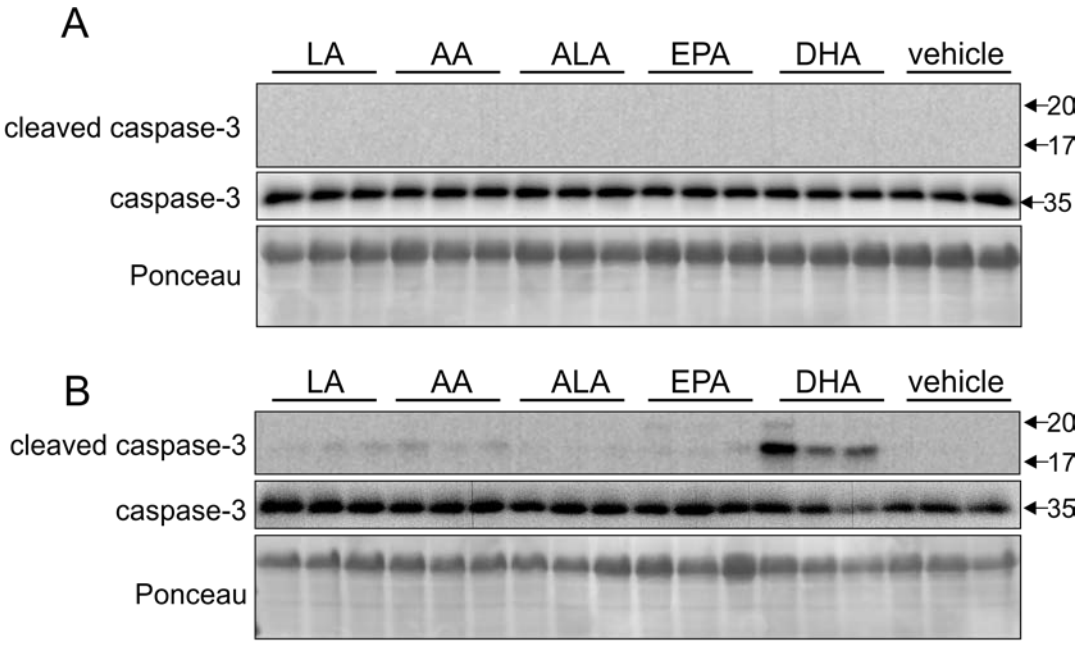

D
D'
E
E'
subconfluent
confluent
Relative Cell Proliferation
EPA
DHA
FA concentration (μM)

Figure 3.9 Effect of ECM substrate and growth state on DNA synthesis of EA.hy926 cells in presence of PUFAs.

EA.hy926 endothelial cells were seeded on plastic culture plates at 9000 cells/cm^2 in the absence (Panel A-E) or presence of Matrigel (Panel A'-E'). Growing cells (day 4) or confluent cells (day 8) were treated with different PUFAs (LA [Panel A, A'], AA [Panel B, B'], ALA [Panel C, C'], EPA [Panel D, D'] and DHA [Panel E, E']) at final concentrations of 1, 5, 20, 40, 60, 80, 100, 125 and 150 μM for 24 hours, at which point, DNA synthesis was assessed using the BrdU cell proliferation assay. Data are normalized with vehicle control values and plotted as means ± SEM (n=3). *Significantly different ($p < 0.05$) from the vehicle control for the respective growth state.

To examine how different PUFA treatments affect proteins associated with cell viability in relation to endothelial growth state, the relative levels of cleaved caspase-3 and caspase-3 were compared in subconfluent and confluent cells grown on Matrigel-coated plates (Figure 3.10A-C). In subconfluent cells, there was no cleaved caspase-3 after various PUFA treatments suggesting the reduction of viability was not through a caspase-3 dependent pathway (Figure 3.10A). In contrast, DHA significantly promoted caspase-3 cleavage in confluent cells indicating a higher level of active caspase-3 (Figure 3.10B).


A
LA
AA
ALA
EPA
DHA
vehicle
cleaved caspase-3
caspase-3
Ponceau
20
17
35
B
LA
AA
ALA
EPA
DHA
vehicle
cleaved caspase-3
caspase-3
Ponceau
20
17
35

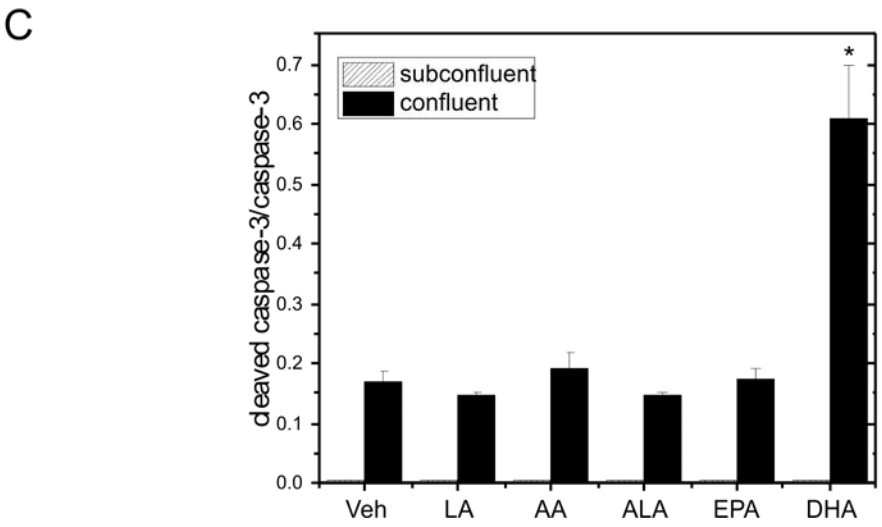

C
subconfluent
confluent
cleaved caspase-3/caspase-3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
*
Veh
LA
AA
ALA
EPA
DHA

Figure 3.10 Effect of PUFA treatment on cleaved caspase-3 and caspase-3 in subconfluent and confluent EA.hy926 cells grown on Matrigel-coated plates.

EA.hy926 endothelial cells were seeded on Matrigel-coated plates at 9000 cells/cm^2 and grown for 4 days. Cells were treated with PUFAs (LA, AA, ALA, EPA and DHA) individually at 125 μM for 8 h. Protein levels of cleaved caspase-3 and caspase-3 were determined by Western blotting of cell lysates prepared after treatments. Representative blots showing 3 lanes per treatment are presented in Panel A (subconfluent) and Panel B (confluent). Densitometry was used to quantify the intensity of the bands in the upper panels. Data are presented as means ± SEM (n=3) for cleaved caspase-3/caspase-3 (Panel C). *Significantly different ($p < 0.05$) from vehicle control.

When examining DNA synthesis, there were differences in the sensitivity of the cells in response to PUFAs. AA (Figure 3.9B and B'), EPA (Figure 3.9D and D'), and DHA (Figure 3.9E and E') decreased the DNA synthesis rate of growing and confluent cells in a dose-dependent manner, while LA (Figure 3.9A and A') and ALA (Figure 3.9C and C') did not. Specifically, when cells were grown on Matrigel-coated plates, AA (Figure 3.9B'), EPA (Figure 3.9D') and DHA (Figure 3.9E') at concentrations higher than 60, 80, and 40 μM, respectively, decreased BrdU incorporation in growing cells, while EPA and DHA higher than 125 and 40 μM, respectively, reduced DNA synthesis in confluent cells. Interestingly, only the highest concentration of AA (150 μM) reduced DNA synthesis of confluent cells grown on Matrigel (Figure 3.9B'). On plastic culture plates, AA (Figure 3.9B), EPA (Figure 3.9D) and DHA (Figure 3.9E) at concentrations higher than 60, 80 and 60 μM, respectively, decreased BrdU incorporation in growing cells, while AA, EPA and DHA higher than 60, 40 and 60 μM, reduced DNA synthesis in confluent cells. It was also observed that treatment with the same concentrations of PUFA caused a greater reduction in DNA synthesis in the absence of Matrigel, especially for AA, EPA and DHA.

To examine how different PUFA treatments affect proteins associated with cell proliferation and endothelial state, the relative levels of MCM2, p-cyclin D1 (T286), cyclin D1 and VE-cadherin were compared in subconfluent (Figure 3.11) and confluent cells (Figure 3.12) grown on Matrigel-coated plates. In subconfluent cells, LA and AA significantly increased MCM2 (Figure 3.11B) while decreasing the p-cyclin D1/cyclin D1 ratio (Figure 3.11C) compared to vehicle control. LA, AA and ALA also significantly increased VE-cadherin levels (Figure 3.11D). In confluent cells, LA, EPA and DHA significantly increased the p-cyclin D1 (T286)/cyclin D1 ratio (Figure 3.12C), but MCM2 (Figure 3.12B) and VE-cadherin (Figure 3.12D) were not changed by these PUFA treatments.

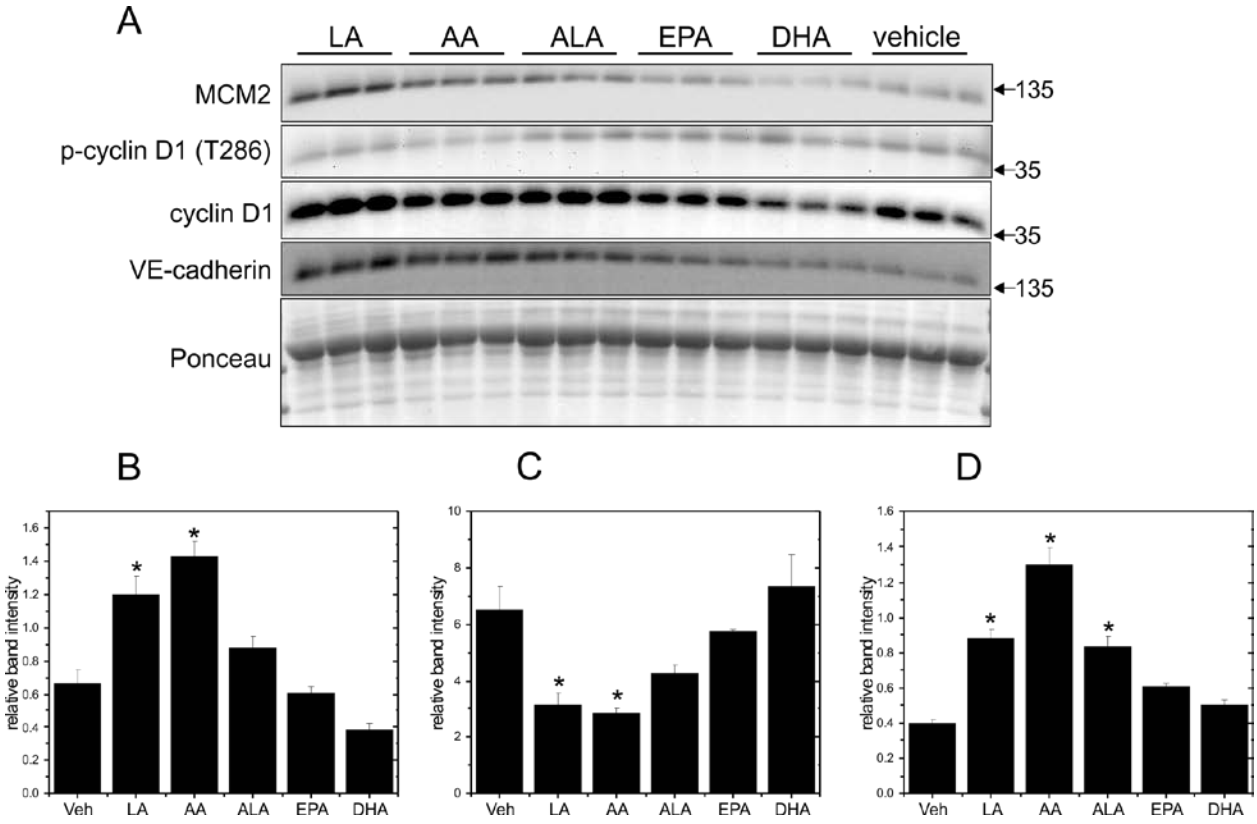

A
LA
AA
ALA
EPA
DHA
vehicle
MCM2
p-cyclin D1 (T286)
cyclin D1
VE-cadherin
Ponceau
135
35
35
135
B
C
D
relative band intensity
Veh
LA
AA
ALA
EPA
DHA

Figure 3.11 Effect of PUFA treatment on MCM2, p-cyclin D1, cyclin D1 and VE-cadherin in subconfluent EA.hy926 cells grown on Matrigel-coated plates.

EA.hy926 endothelial cells were seeded on Matrigel-coated plates at 9000 cells/cm^2 and grown for 4 days. Cells were treated with PUFAs (LA, AA, ALA, EPA and DHA) individually at 125 μM for 8 h. Protein levels of MCM2, p-cyclinD1, cyclin D1 and VE-cadherin were determined by Western blotting of cell lysates prepared after treatments. Representative blots showing 3 lanes per treatment are presented in Panel A. Densitometry was used to quantify the intensity of the bands in the upper panel and data were normalized to a band visualized by Ponceau staining. Data are presented as means ± SEM (n=3) for MCM2 (Panel B), p-cyclin D1/cyclin D1 (Panel C) and VE-cadherin (Panel D). *Significantly different ($p < 0.05$) from vehicle control.

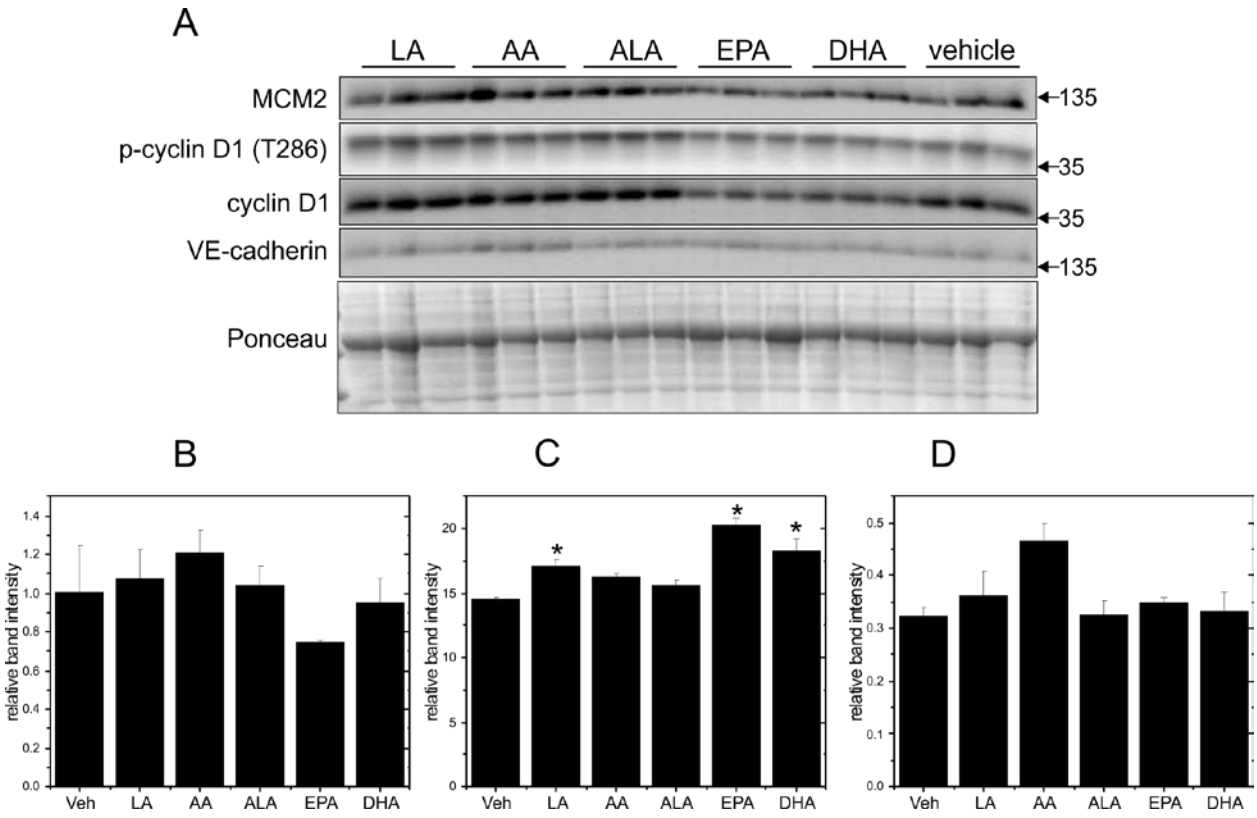


Figure 3.12 Effect of PUFA treatment on MCM2, p-cyclin D1, cyclin D1 and VE-cadherin in confluent EA.hy926 cells grown on Matrigel-coated plates.

EA.hy926 endothelial cells were seeded on Matrigel-coated plates at 9000 cells/cm^2 and grown for 8 days. Cells were treated with PUFAs (LA, AA, ALA, EPA and DHA) individually at 125 μM for 8 h. Protein levels of MCM2, p-cyclinD1, cyclin D1 and VE-cadherin were determined by Western blotting of cell lysates prepared after treatments. Representative blots showing 3 lanes per treatment are presented in Panel A. Densitometry was used to quantify the intensity of the bands in the upper panel and data were normalized to a band visualized by Ponceau staining. Data are presented as means ± SEM (n=3) for MCM2 (Panel B), p-cyclin D1/cyclin D1 (Panel C) and VE-cadherin (Panel D). *Significantly different ($p < 0.05$) from vehicle control.

3.7 Discussion

The present study is the first to show that EA.hy926 endothelial cells enter a quiescent state after reaching confluence when grown on Matrigel. VE-cadherin is important for maintaining and controlling of endothelial intercellular contacts, and its expression suggests the presence of cell junctions, which only occurs once a confluent monolayer is formed. This interpretation is possible because there is a decrease in the DNA synthesis rate as a result of contact inhibition and there is no loss of cell viability. Furthermore, there was a decline in MCM2 levels and the p-cyclin D1/cyclin D1 ratio, which indicates the cells had left the cell cycle. The high levels of p27kip1 across all time points supported the evidence that cell cycle progression to S phase is inhibited. In contrast, cells grown either without Matrigel or with collagen do not become quiescent, which is indicated by a continued high level of BrdU incorporation after confluence is reached. The high p-cyclin D1/cyclin D1 ratio and VE-cadherin levels with a low level of p27kip1 in cells grown on plastic also support the view the cells remain in the cell cycle even after cell-to-cell contacts have formed. Quiescent cells are also characterized by low levels of apoptosis and senescence. In the current study, cells were cultured for up to 12 days without changing the media. Accumulation of waste metabolites and deprivation of nutrients can lead to cell death due to apoptosis and cessation of growth due to senescence. However, a comparison of caspase-3 activity, which is an indicator for apoptosis, and SA-β-galactosidase activity, which is a known biomarker of senescent cells, has shown that cells grown on Matrigel have less potential to become either apoptotic or senescent. Thus, the evidence demonstrates that cells grown on Matrigel-coated plates are more likely to be quiescent than cells grown on plastic plates. Additionally, this study has shown that confluent cells are protected from high concentrations of PUFAs, since treatment with these compounds does not reduce viability regardless of whether they are grown on Matrigel or not. However, Matrigel

promotes the viability of subconfluent cells exposed to PUFAs, specifically AA, EPA and DHA, while the precursor C18 LA and ALA have no negative effect on viability at even the highest concentration employed. Interestingly, DHA induced cleavage of caspase-3 in confluent cells but not subconfluent cells, which suggests that the effect of DHA on cell viability potentially involves an apoptotic mechanism that only operates in the quiescent state. Finally, AA, EPA and DHA have similar effects on BrdU incorporation in subconfluent and confluent cells, albeit with different sensitivities in relation to concentration, while the precursor PUFAs do not impact negatively on proliferation. Specifically, at higher concentrations, these PUFAs induce cell quiescence by suppressing DNA synthesis. Our results have thus established the critical importance of both ECM substrate and growth state in how endothelial cells respond to dietary factors such as PUFAs and have shown how specific PUFAs could potentially influence endothelial function in both healthy and pathological conditions when taken at a high dose such as in supplements.

Under physiological conditions, the endothelial cells present on the inner surface of blood vessels are in their resting, non-dividing state. However, endothelial cells have an intrinsic ability to exit this quiescent state and proliferate, which is typically seen in cell culture. But can these cells re-enter the quiescent state when they become confluent as they are in a healthy vessel? Our BrdU assay results showed that EA.hy926 endothelial cells become quiescent after confluence is reached as indicated by a decrease in the DNA synthesis rate, decrease of MCM2 expression and reduced p-cyclin D1/cyclin D1 ratio only when grown on a specific ECM substrate, namely Matrigel. Furthermore, the cell number data show that quiescence does not occur because of a loss of cell viability, thus implicating contact inhibition in the observed reduction of DNA synthesis. Interestingly, the cells do not become quiescent even after becoming confluent when

grown on collagen-coated or uncoated plates. As a result, our study is the first to show that a thin layer coating of Matrigel provides better cellular attachment, thus enabling entry into a quiescent state *in vitro*, which is a more representative model of endothelial cell characteristics and responses *in vivo*. At the same time, it is noteworthy that no study has used Matrigel as an ECM substrate to grow EA.hy926 endothelial cells and we have demonstrated its presence protects the viability of cells against the effects of higher concentrations of PUFAs (AA, EPA and DHA). Although a few studies have used other ECM substrates such as gelatin [14-16] and fibronectin [17] to test the viability of endothelial cells in response to PUFA treatment, comparisons with non-coated plates were lacking.

The importance of growth state has received limited attention with respect to the endothelial cell response to PUFAs. We have shown that confluent cells have higher viability than growing cells in the presence of higher concentrations of PUFAs, specifically AA, EPA and DHA. In support of our findings, the viability of confluent HUVECs [18], confluent primary porcine pulmonary artery endothelial cells [14], confluent primary human saphenous vein endothelial cells [17], and confluent and post-confluent EA.hy926 endothelial cells [19] were not affected by treatment with various PUFAs, while the viability of proliferating HUVECs [15, 18, 19] was reduced. There are two studies showing that viability of 70-90% confluent EA.hy926 [20] and HUVECs [21] was not affected by treatment with 100 μM DHA for 24 h and 8 h, respectively. Although the cells were not 100% confluent, the establishment of cellular contact as seen in cell cultures nearing confluency is still sufficient to help survival. Studies that did not indicate the growth state of the endothelial cells are excluded from the discussions here [22-24].

The range of fatty acid concentrations in the present study was chosen to be representative of the circulating free fatty acid concentrations that would be in contact with the endothelium *in*

vivo [5]. We observed that viability and proliferation of growing endothelial cells was more sensitive to C20 and C22 PUFAs (AA, EPA and DHA) than their precursor PUFAs (LA and ALA, which are the two dietary essential fatty acids), regardless of whether they are omega-6 or omega-3 fatty acids. Cell viability was decreased when AA was >20 μM, EPA >5 μM, and DHA > 60 μM in growing endothelial cells in the absence of Matrigel, whereas 150 μM of LA or ALA for 24 h had no effect on viability. Likewise, Spector et al. [25] reported that treatment with LA (150 μM for 6 h) does not affect the viability of growing primary HUVECs, while Kim et al. [18] found that that 40 μM DHA induced death in growing cells in a time-dependent manner. Gousset-Dupont et al. [19] reported 40 μM of EPA and DHA reduced viability of growing EA.hy926 endothelial cells, which compares favourably with our observations that EPA and DHA were lethal to cells at 5 μM and 60 μM, respectively. Concentrations higher than 150 μM and incubation times longer than 24 h were not tested in our experiments, but in other studies 300 μM LA or AA for 24 hours induced apoptosis of growing primary HUVECs, with further increases in apoptosis when the incubation time was extended to 48 h [15]. We did not examine FA concentrations above 150 μM since total non-esterified FA levels in plasma are approximately 300 μM in healthy individuals [5]. Of that total, LA represents approximately 25% [26] or about 75 μM. Furthermore, LA is substantially higher than the other PUFAs, which range from 3-8 μM in their non-esterified form [26].

In the present study, DNA synthesis of both growing and confluent cells grown on Matrigel-coated and plastic plates was reduced in a dose-dependent manner, particularly by the elongated PUFAs (AA, EPA, DHA). Interestingly, AA slightly lowered the DNA synthesis rate of confluent cells grown on Matrigel-coated plates but only at the highest concentration (150 μM) tested in these cultures. The data presented by Kim et al. [18] are in agreement with our results

showing 40 μM DHA decreased proliferation of growing HUVECs after 24 h, while 40 μM AA did not affect proliferation of either growing or confluent cells. However, Kim et al. [18] also showed that 40 μM DHA did not decrease the proliferation of confluent HUVECs after 24 h, whereas we observed the opposite. On the other hand, Artwohl et al. [15] showed that treatment of growing HUVECs with 300 μM LA and AA for 48 h caused cell cycle arrest. Although their experimental conditions (concentration and time length) went beyond what we employed, we did find that, after 24 hours, 150 μM of LA did not affect proliferation while AA at concentrations higher than 60 μM decreased DNA synthesis in growing cells. Although absolute uniformity between these studies does not exist, it is apparent that certain concentrations of DHA and AA do consistently slow the proliferation rate of growing cells.

We have also explored potential mechanisms of how different PUFAs affect the viability and proliferation of growing and confluent cells by comparing specific proteins associated with cell apoptosis including cleaved caspase-3 and caspase-3, and proteins associated with proliferation (MCM2, p-cyclin D1, cyclin D1) as well as VE-cadherin, which is an endothelial-specific junction molecule important in establishing contact inhibition and thus controlling proliferation [27]. We observed an increase of cleaved caspase-3 in confluent cells grown on Matrigel by DHA but not other PUFAs, while active caspase-3 was not seen in subconfluent cells. This suggests that the reduction in viability seen with DHA treatment of both growing and confluent cells occurs by different mechanisms. The differential effects of DHA on apoptosis may be due to differences in the receptors present on the cells when in different growth states. Furthermore, this is the first time activation of caspase-3 by cleavage has been reported in confluent human endothelial cells in response to direct DHA treatment. Artwohl et al. [28] found caspase-8 activation is associated with apoptosis induced by 300 μM of LA, AA and ALA on

HUVECs, human aortic endothelial cells, human retinal endothelial cells and endothelial progenitor cells. Artwohl [15] also found 300 μM of LA and AA induced apoptosis of HUVECs by reducing apoptosis inhibitor bcl-2 and increasing levels of bak expression. Meerarani et al. [29] have reported an up-regulation of caspase-3 by 90 μM LA on porcine pulmonary arterial endothelial cells. In terms of the changes of proliferation related molecules by different PUFAs, our results indicate that indirect modulation of the cellular machinery that regulates cell proliferation by AA, EPA and DHA treatment is the reason these PUFAs reduce DNA synthesis. However, other possible mechanisms operating outside of the G1 phase of the cell cycle were not examined in this study. Few studies have looked at these molecules when investigating the effect of PUFAs on endothelial cell proliferation. Su et al. [30] showed high concentrations of free fatty acids affect the expression of cell cycle-related proteins such as cyclin B, cyclin D, cyclin E, p53, and cell division cycle protein 14 in human brain vascular endothelial cells, although the fatty acid composition of their treatment was not specified. Artwohl et al. [15] showed a correlation between an increase in the expression of p21 (inhibitor of cyclin-dependent kinase), a decrease of cyclin D3 protein expression, an increased number of cells in G_0/G_1 phase, and a decreased number of cells in S phase when they reported LA and AA caused cell cycle arrest in HUVECs, and suggested a sophisticated cell cycle controlling mechanisms. When Kim et al. [18] reported exposure for 24 h with 40 μM DHA decreased the proliferation of HUVECs, they also indicated there was an increase of cells in the sub G_1 phase. Given this uncertainty in how they operate, the mechanisms by which AA, EPA and DHA reduce DNA synthesis still require further investigation.

Cell preparation before treatment may be a factor affecting the cellular response to PUFAs. We have shown that up to 60 μM AA does not increase proliferation and may slightly decrease

proliferation of subconfluent cells. On the other hand, Fiorio et al. [16] showed that adding 5 μM AA to growing breast lobular-infiltrating carcinoma (B-TEC) derived endothelial cells for 24 hours increased proliferation (according to cell number) compared to control, and that 2 μM AA significantly increased the number of BAECs compared to 5% FBS alone [31]. It is worth pointing out that in the studies by Fiorio et al. [16, 31], cells were starved in the presence of low serum concentration (1%) for 24 h before adding AA. Given this experimental design, AA could be stimulating the proliferation of serum-starved cells. We believe that adding PUFAs to the original media instead of to pre-starved cells has greater physiological relevance regardless of the source of the endothelial cells.

Studies on how PUFAs can modulate endothelial function are of great importance because on one hand, healthy endothelial cells are vital in maintaining cardiovascular homeostasis while on the other hand, endothelial dysfunction promotes the development and progression of vascular disease [32]. Dietary PUFAs could modulate various aspects of vascular function in both healthy and dysfunctional endothelial cells. Consumption of n-3 PUFA is associated with reduced CVD risk [33] and it is proposed that the beneficial effects of n-3 PUFA may be due to their effects on endothelial cell function [34]. Our study is the first to show how different PUFAs, including n-6 PUFA (LA and AA) and n-3 PUFA (ALA, EPA and DHA), impact endothelial cells differently in terms of viability and proliferation. Further studies are still required to determine the effects of PUFAs on other functions of endothelial cells such as migration and barrier function.

3.8 Conclusion

In conclusion, our results have shown that extracellular matrix proteins, which can modulate growth state, are important for the EA.hy926 endothelial cell response to different PUFAs in terms of viability and proliferation. This observation may also apply to other dietary

factors, but studies examining this possibility have not been done. Higher concentrations of PUFA, especially AA (>20 μM), EPA (>5 μM) and DHA (>60 μM), reduce cell viability, particularly when the cells are not grown on an ECM substrate. However, confluent endothelial cells that become quiescent due to contact inhibition, which is the state that represents the normal endothelial cell monolayer in a healthy vessel, are more resistance to high concentrations of PUFAs than subconfluent, growing cells. Another unique finding of this study is DHA treatment significantly induced cleaved caspase-3 in quiescent endothelial cells, which are representative of the normal healthy endothelium. Further investigation is therefore warranted to determine the clinical significance of this observation as it applies to the physiological and pathological conditions of endothelial cells in the vasculature. We have also shown that AA, EPA and DHA, but not their precursor fatty acids (LA and ALA), reduce DNA synthesis of EA.hy926 endothelial cells grown on Matrigel-coated and plastic plates in a dose-dependent manner. However, whether these responses to PUFAs are favourable or detrimental to endothelial cells and overall vascular function will depend on whether cells are healthy or dysfunctional, and these conditions warrant further investigation.

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Chapter 4: Apoptosis of EA.hy926 Endothelial Cells in Response to Docosahexaenoic Acid (DHA) Requires Growth State-Dependent Activation of Caspase-3 and MAPK

4.1 Contribution Statement

I, Youjia Du, made the following contribution to this study:

- Developed the study concept and experimental design with other contributors
- Conducted all experiments and data analysis
- Wrote the manuscript
- Interpreted results and edited the manuscript with other contributors

Other contributors:

- Peter Zahradka
- Carla G. Taylor
- Harold M. Aukema

4.2 Study Rationale

In the previous chapter, it was shown that EA.hy926 endothelial cells become quiescent after reaching confluence when grown on Matrigel-coated plates compared to cells grown on collagen-coated or plastic plates. Since endothelial cell quiescence is vital for expressing the phenotype associated with confluent endothelial cells, it is important to mimic the typical basal state present in the endothelium of healthy blood vessels. Therefore, Matrigel was used as a substrate to grow EA.hy926 endothelial cells in all subsequent studies. Chapter 3 also showed that when cells were grown on plastic plates, the viability of confluent cells was less sensitive to higher concentrations of AA (Figure 3.8B), EPA (Figure 3.8D) and DHA (Figure 3.8E) than subconfluent cells, and this observation further emphasized the importance of growth state (subconfluent, confluent) in the endothelial cell response to PUFAs. Results from Chapter 3 also suggested that among the PUFAs that had a negative effect on cells (AA, EPA and DHA), DHA was the most potent. Furthermore, DHA induced caspase-3 activation in confluent but not growing EA.hy926 endothelial cells (Figure 3.10). Given the key role of caspase-3 in mediating one of the primary cell death pathways, apoptosis, further studies on whether DHA induced apoptosis in confluent cells as well as the mechanism of action of DHA on endothelial cells in different growth states were needed. MAPKs (p38 MPAK, ERK1/2 and JNK) are kinases that transduce signals from the extracellular environment to the intracellular space, thus regulating a variety of cellular responses including cell survival and apoptosis. However, whether the action of DHA on both subconfluent and confluent EA.hy926 cells was mediated by MAPKs has received no attention to date. Therefore, the experiments described in this chapter were designed to answer these questions by first examining whether DHA induced apoptosis in confluent EA.hy926 endothelial cells, and second, determining whether this regulation was achieved through activation of specific caspase and MAPK family members.

4.3 Abstract

Endothelial cells, which help to maintain vascular homeostasis, can be functionally modulated by polyunsaturated fatty acids. Previously, we reported that DHA reduced the viability of both growing and confluent EA.hy926 endothelial cells in a dose-dependent manner, but caspase-3 activation was observed only in confluent cells. This study therefore examined the molecular mechanism by which DHA affects the viability of confluent cells, with a focus on the interaction between caspase-3 and MAPK. Since our previous finding showed DHA promoted caspase-3 activation, we examined whether DHA also induced apoptosis. Our results revealed apoptosis occurred only in confluent (quiescent) but not subconfluent (growing) EA.hy926 endothelial cells. Apoptosis was not through the extrinsic pathway but it was mitochondria-dependent and required both caspase-3 and p38 MAPK. When phosphorylation of various MAPKs (p38, ERK1/2 and JNK) was monitored in DHA-treated subconfluent and confluent endothelial cells, distinct growth state-dependent patterns of activation were seen over 8 hours. p38 MAPK was activated at 10 min and 4 h in growing cells, but ERK1/2 and JNK were only activated at 4 h. In confluent cells, DHA stimulated p38 MAPK at 10 min, while ERK1/2 and JNK phosphorylation occurred at both 10 min and 4 h. Additionally, we examined DUSP1 and found its activation was involved in the negative regulation of p38 MAPK, while MKK3/MKK6 parallels with p38 MAPK activation. A positive regulatory loop was also found between p38 MAPK and caspase-3. However, this regulation on p38 MAPK was achieved neither through caspase-3 activity on MKK3, which phosphorylates p38 MAPK, or DUSP1. These results suggest that growth state plays an important role in modulating the endothelial cell response to DHA via caspase-3 and MAPK.

4.4 Introduction

Endothelial cells, which form the endothelium that covers the inner surface of blood vessels, play important roles in maintaining vascular homeostasis. While endothelial cells were originally considered merely a barrier that separates blood from tissues, in 1891 Heidenhahn demonstrated the endothelium was an active secretory system [1]. Currently, endothelial cells are recognized for their roles in blood vessel formation, secretion of vasodilators (e.g. NO and PGI_2) and vasoconstrictors (e.g. endothelin), the inflammatory response, coagulation and fibrinolysis [2-9].

Elevated levels of FFA have been documented in atherosclerosis and T2DM [10, 11], with FFA reaching 300 μM in healthy individuals and higher in persons with type 2 diabetes [12]. PUFAs, especially n-3 PUFAs, have been shown in numerous dietary intervention studies to exert beneficial effects on endothelial cell function as measured by flow-mediated dilatation [13], a method that measures contractility. However, PUFAs have also been shown to up-regulate the inflammatory response and induce apoptosis in various human endothelial cell types [14, 15]. Thus, PUFAs appear to have both positive and negative effects on vascular cells.

Endothelial cells in healthy blood vessels are confluent and in a quiescent state. Once activated, endothelial cells can migrate and proliferate, a process that allows the cells to participate in inflammation, wound repair, and blood vessel formation when required. Although a healthy vasculature requires viable and functional endothelial cells, it may also allow cell death for removal of dysfunctional cells and for cell turnover. Apoptosis is a type of cell death that plays important roles in angiogenesis, regression, and vessel remodeling and it has been suggested as a potential therapeutic target for neovascularization in tumors [16, 17]. Dysregulation of endothelial cell apoptosis has been implicated in vascular diseases. On one hand, apoptotic endothelial cells resulting from damage contribute to the development of

atherosclerosis; on the other hand, endothelial cells exhibit higher levels of apoptosis than regeneration in atherosclerotic plaque, which results in dysfunctional endothelium [18].

Interestingly, the importance of growth state (growing vs quiescent) in the endothelial cell response to PUFAs has not received much attention. Previously, we reported that PUFAs (LA, AA, ALA, EPA and DHA) differentially impact growing and confluent EA.hy926 endothelial cell viability [19]. We have also observed that DHA decreased the viability of both growing and confluent endothelial cells grown on Matrigel-coated plates [19], but induced caspase-3 activation only in confluent cells [19]. These data suggested that modulation of apoptosis by DHA was dependent upon the growth state of the cells, however, we did not investigate whether activation of caspase-3 was via the extrinsic or intrinsic pathway. The extrinsic pathway requires a series of events including the binding of ligands to the death receptor that leads to formation of Death Induced Signaling complex, caspase-8 activation, and finally capase-3 activation which induces apoptosis [20]. The intrinsic pathway, also called mitochondria-dependent apoptosis, involves disruption of mitochondria membrane permeability, release of cytochrome c, and activation of caspase-9 that leads to caspase-3 activation and thus apoptosis [21, 22].

MAPK, which include p38, ERK1/2 and JNK, are serine-threonine kinases that transduce extracellular signals, such as cellular stress, pro-inflammatory cytokines and mitogenic stimuli, into intracellular signals. MAPK pathways are involved in various cellular events including cell survival and apoptosis [23], and they are responsive to DHA in various cell types [24-26]. DUSP1 is a negative regulator of MAPKs including p38 MAPK [27].

In this study, we hypothesized that DHA induces apoptosis in confluent endothelial cells through caspase-3 and MAPK dependent pathways. The reduction of cell viability by DHA in growing and confluent endothelial cells was examined in relation to the activation state of different MAPKs. As part of this investigation, we also investigated the role of MKK3/MKK6

and DUSP1 in p38 MAPK activation, as well as the cross-talk between p38 MAPK and caspase-3 using specific inhibitors.

4.5 Materials and Methods

Cell culture

EA.hy926 endothelial cells were purchased from ATCC (cat. CRL 2922) and propagated as described previously [19]. EA.hy926 endothelial cells were seeded at a density of 9000 cells/cm^2. The day cells were seeded was referred to as day 0. Subconfluent cells were used at day 4, and confluent cells were used at day 8. EA.hy926 endothelial cells were grown on Matrigel-coated plates, prepared as previously described [19], for up to 8 days. Cells were used within 20 passages.

Cell treatments

Polyunsaturated fatty acids (LA, AA, ALA, EPA and DHA; Cayman Chemical) were dissolved in ethanol and then mixed with 5% fatty acid-free bovine serum albumin in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). They were added (without disturbing the cells) to subconfluent and confluent cells for up to 8 h. BCI (dual specificity protein phosphatase 1/6 inhibitor, Millipore, #317496), SB202190 (a p38 MAPK inhibitor, Tocris, #1264), Z-D(OMe)-E(OMe)-V-D(OMe)-FMK (Z-DEVD-FMK) (a caspase-3 inhibitor, R&D System, #FMK004), Z-I-E(OMe)-T-D(OMe)-FMK (Z-IETD-FMK) (a caspase-8 inhibitor, NOVUS, #NBP2-29397) were dissolved in DMSO and used at final concentrations of 5 μM, 1μM, 20 μM and 20 μM, respectively. BCI, Z-DEV-FMK and Z-IETD-FMK were pre-incubated with cells for 0 min or 30 min before adding DHA. SB202190 was added 30 min before DHA treatment or 2 h after DHA treatment.

Apoptosis assay

Confluent endothelial cells grown on 96 well plates were treated with 125 μM DHA for 24 h. Apoptosis was detected using the Apoptosis/Necrosis Detection Kit (blue, green, red, abcam, Product No. 176749). Cells were washed with the Assay Buffer provided in the kit followed by addition of Apopxin Green Indicator, 7-aminoactinomycin D (7-AAD), and Cytocalcein violet 450 to the cells. Cells were incubated for 60 min followed by a wash with the Assay Buffer, then imaged with a fluorescence microscope under excitation/emission wavelengths of 490/525 nm to detect apoptotic cells (green), 550/650 nm to detect live cells (blue) and 405/450 nm to detect the later stage of apoptosis and necrotic cells (red). Images were taken at a magnification of 100×.

Western blotting

After various treatments, subconfluent and confluent EA.hy926 cells were lysed with 2× sample buffer (125 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate, 20% glycerol) and briefly sonicated. Twenty μg of lysate protein was subjected to SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to PVDF membranes for immunoblotting. Primary antibodies were diluted in TBST (Tris-buffered saline with Tween-20; 150 mM NaCl, 20 mM Tris pH 7.4, 0.05% Tween 20) containing 3% BSA. The primary antibodies employed were rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (1:1000, Cell Signaling, #9211), rabbit anti-p38 MAPK (1:1000, Cell Signaling, #9212), rabbit anti-phospho-p44/42 MAPK (also named extracellular signal regulated kinase, ERK) (Thr202/Try204) (1:1000, Cell Signaling, #9101), rabbit anti-p44/42 MAPK (1:1000, Cell Signaling, #9102), rabbit anti-phospho-JNK (Thr183/Try185) (1:1000, Cell Signaling, #9251), rabbit anti-JNK (1:1000, Cell Signaling, #9252), rabbit anti-phospho-DUSP1 (Ser359) (1:1000, Cell Signaling, #2857), rabbit anti-DUSP1 (1:1000, Abcam, #138265), rabbit anti-phospho-MKK3 (Ser189)/MKK6 (Ser207) (1:1000, Cell Signaling, #9231), rabbit anti-MKK3 (D4C3) (1:1000, Cell Signaling, #8535), rabbit anti-cleaved caspase-3 (1:1000, Cell Signaling, #9664), rabbit anti-caspase-3 (1:1000, Cell

Signaling, #9662), rabbit anti-cleaved caspase-9 (1:1000, Cell Signaling, #7237), and rabbit anti-caspase-9 (human specific) (1:1000, Cell Signaling, #9502). HRP conjugated secondary antibodies were used at a dilution of 1:10000 in TBST containing 1% BSA. The relative band intensities were captured using a FluorChem® Q imager and quantified with AlphaView software (Alpha Innotech). The Western blot data were normalized to a selected band on a Ponceau S stained image of the blot.

Statistical Analysis

One-way ANOVA and post-hoc testing with Tukey's test or Student's t test were used to assess statistical significance, which was set at $p<0.05$ (SPSS version 20). All experiments were independently repeated at least 3 times. Within each experiment, the plotted values were derived from treatments done in triplicate (n=3) and are expressed as mean ± SEM.

4.6 Results

Induction of apoptosis by DHA

We previously found that DHA induced caspase-3 activation in confluent cells [19], however, we did not directly establish that DHA induced apoptosis. We therefore stained confluent DHA-treated endothelial cells with Apopoxin Green Indicator. This assay confirmed that these cells became apoptotic in response to DHA treatment, in agreement with the indirect evidence provided by caspase-3 activation (Figure 4.1).

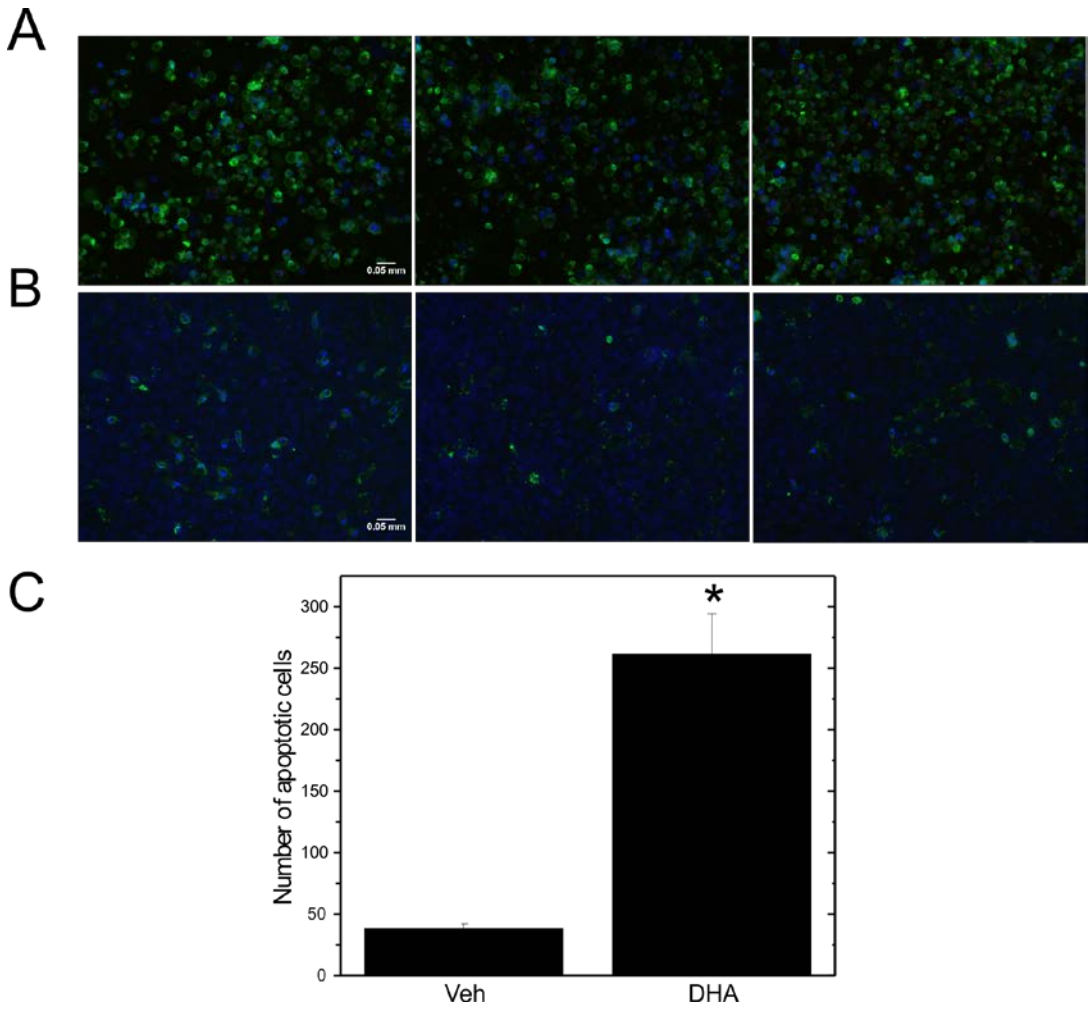


Figure 4.1 Apoptosis assay.

Confluent EA.hy926 endothelial cells were treated with 125 μM DHA (Panel A) or vehicle control (Panel B) for 24 h. Apopxin Green Indicator, 7-AAD, and Cytocalcein violet 450 were added to the cells to stain apoptotic (green), live (blue) and later stage apoptotic/necrotic cells (red), respectively. Three representative images per treatment are shown. Quantification of the apoptotic cells in the images is presented in Panel C as means ± SEM (n=3). *Significantly different ($p<0.05$) from Veh. Veh - vehicle.

Caspase-3 activation in confluent cells

Our previous study showed that cleaved caspase-3 was present in confluent endothelial cells 8 h after DHA treatment [19]. Since it was not possible to establish when caspase-3 cleavage occurred, the presence of cleaved caspase-3 was monitored over a time course extending from 0 to 8 hours. This experiment showed that activation of caspase-3 could be detected as early as 4 h after DHA treatment (Figure 4.2).

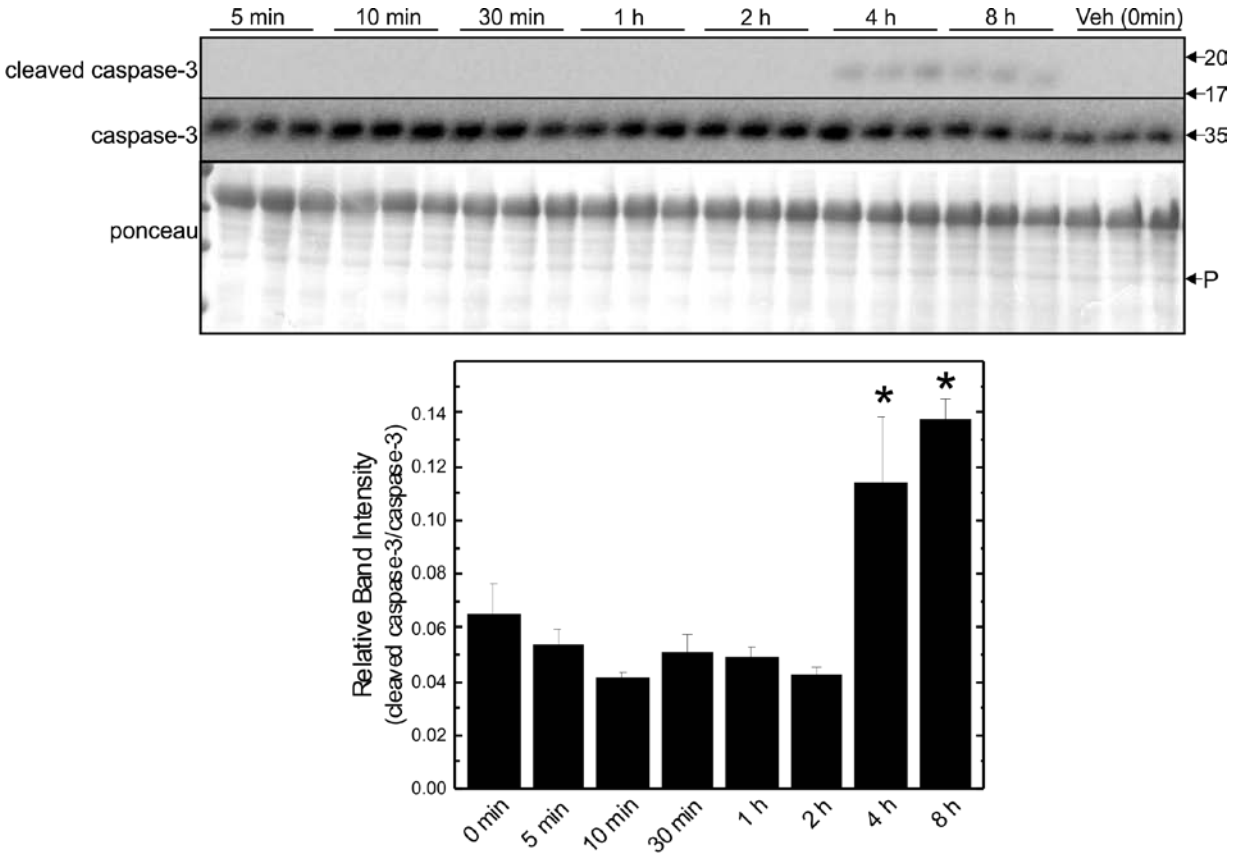


Figure 4.2 Caspase-3 activation in DHA-treated confluent cells.

Confluent EA.hy926 endothelial cells were treated for various times (0 min - 8 h) with 125 μM DHA. Levels of cleaved caspase-3 and caspase-3 were determined by Western blotting of cell lysates. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in the upper Panel. Data are presented in the lower Panel as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. Veh - vehicle.

Activation patterns of MAPKs in response to DHA differ in subconfluent and confluent cells

Cleavage of caspase-3 occurs after stimulation of several intracellular signaling pathways, including MAPKs [28, 29], which are activated by extracellular stimuli and transduce signals within the cell to induce an intracellular response. MAPKs are involved with various cellular activities such as survival, proliferation, migration, and differentiation, as well as apoptosis [30, 31]. To examine whether MAPKs are activated by DHA in our experimental conditions, we first investigated MAPK activation upon DHA treatment in growing (Figure 4.3A) and confluent (Figure 4.3B) endothelial cells. In growing cells, DHA activated p38MAPK at 5 min and 10 min, and at 4 h and 8 h, but only activated ERK1/2 and JNK at 4 h and 8 h. Conversely, DHA treatment of confluent cells stimulated p38MAPK at 5 and 10 min, while ERK1/2 and JNK phosphorylation occurred at both early (5 and 10 min) and later time points (4 and 8 h). These results show that activation of individual MAPKs follow distinct temporal patterns that are determined by these two growth states in endothelial cells.

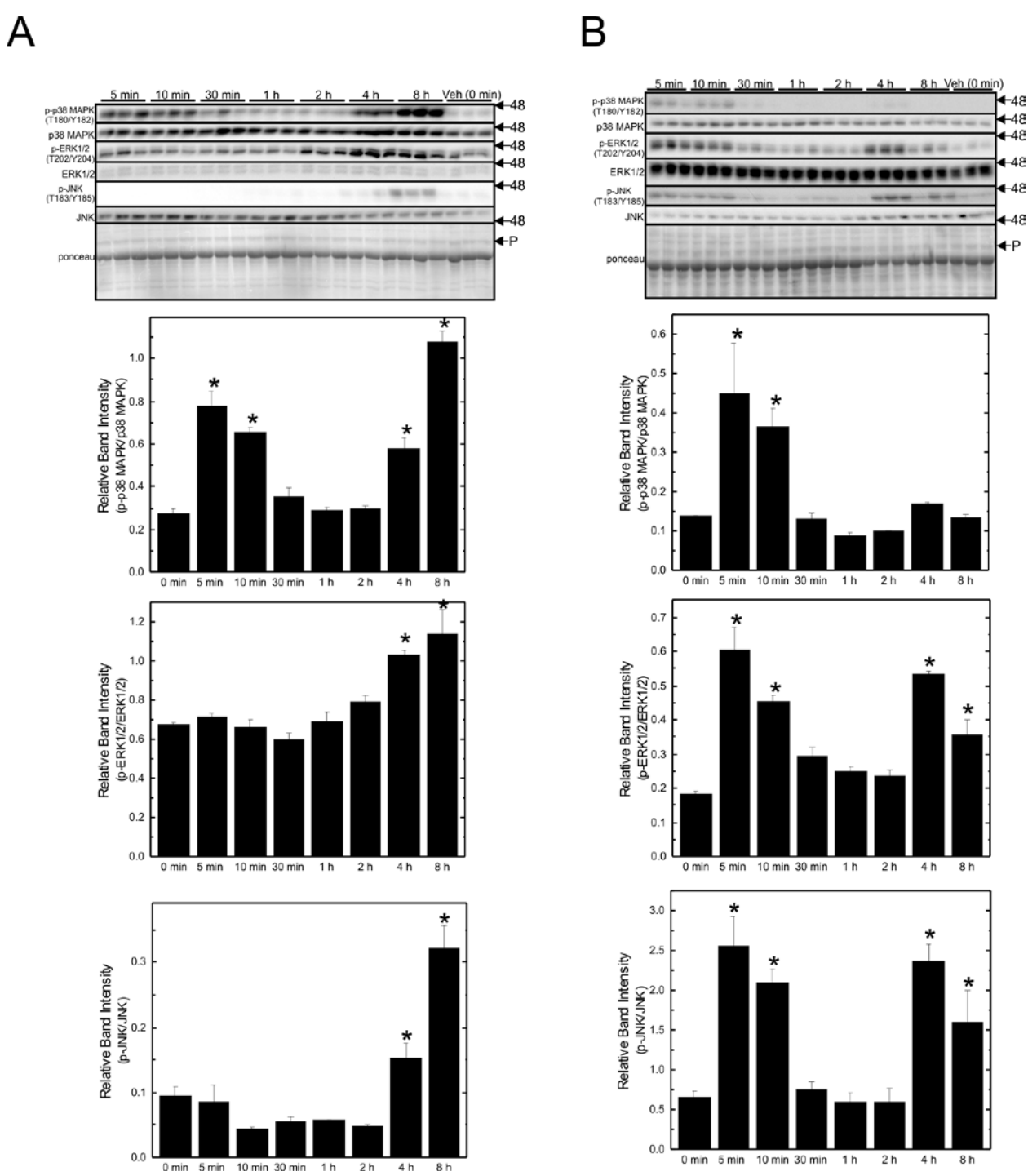

A
B
5 min
10 min
30 min
1 h
2 h
4 h
8 h
Veh (0 min)
p-p38 MAPK (T180/Y182)
p38 MAPK
p-ERK1/2 (T202/Y204)
ERK1/2
p-JNK (T183/Y185)
JNK
ponceau
48
P
Relative Band Intensity (p-p38 MAPK/p38 MAPK)
Relative Band Intensity (p-ERK1/2/ERK1/2)
Relative Band Intensity (p-JNK/JNK)
0 min
5 min
10 min
30 min
1 h
2 h
4 h
8 h

Figure 4.3 MAPK activation by DHA in subconfluent and confluent cells.

Subconfluent (Panel A) and confluent (Panel B) EA.hy926 endothelial cells were treated with 125 μM DHA for various times (0 min - 8 h). Levels of p-p38 MAPK, p38 MAPK, p-ERK1/2, ERK1/2, p-JNK and JNK were determined by Western blotting of cell lysates prepared after treatment at various time points (0 min, 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h). Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in the upper Panels. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented in the lower Panels as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. Veh - vehicle.

Pattern of p38 MAPK activation by DHA is unique compared to other PUFA (LA, AA, ALA, EPA) treatments

To examine whether other PUFAs activated p38 MAPK, subconfluent cells were treated with 125 μM LA, AA, ALA or EPA for up to 8 h. All of the PUFAs (LA [Figure 4.4A], AA [Figure 4.4B], ALA [Figure 4.4C], EPA [Figure 4.4D] and DHA [Figure 4.4E]) transiently activated p38 MAPK at 5 and 10 min, but only DHA (Figure 4.4E) also induced activation of p38 MAPK at 4 and 8 h. These results indicated that the pattern of p38 MAPK activation by DHA in growing cells is unique among other PUFAs (LA, AA, ALA, EPA).

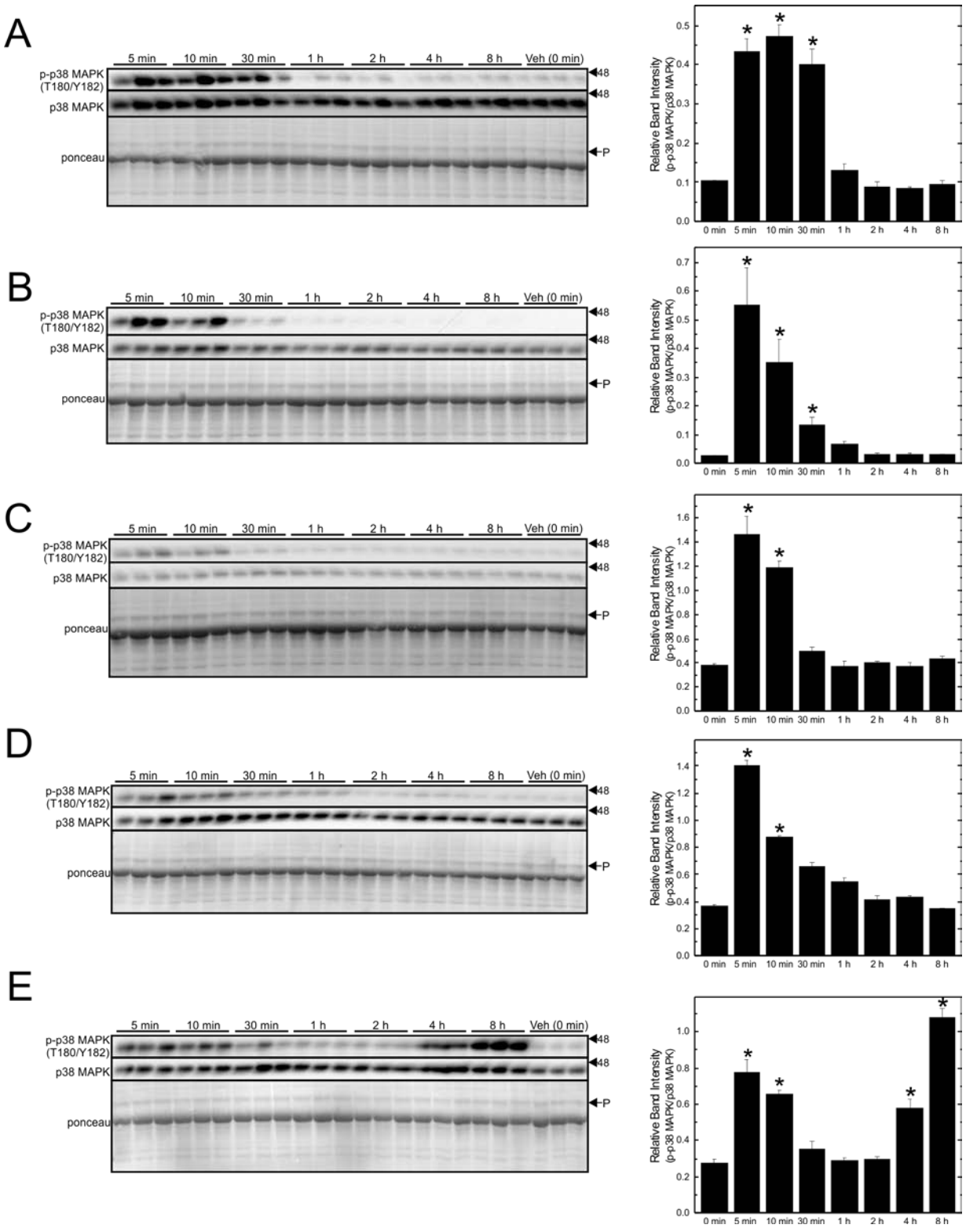

A
B
C
D
E
5 min
10 min
30 min
1 h
2 h
4 h
8 h
Veh (0 min)
p-p38 MAPK (T180/Y182)
p38 MAPK
ponceau
48
48
P
Relative Band Intensity (p-p38 MAPK/p38 MAPK)
0 min
5 min
10 min
30 min
1 h
2 h
4 h
8 h

Figure 4.4 Patterns of p38 MAPK activation by DHA compared to other PUFAs (LA, AA, ALA, EPA).

Subconfluent EA.hy926 endothelial cells were treated with 125 μM LA (Panel A), AA (Panel B), ALA (Panel C), EPA (Panel D) or DHA (Panel E) for various times (0 min - 8 h). Levels of p-p38 MAPK and p38 MAPK were determined by Western blotting of cell lysates prepared after treatment for various times (0 min, 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h). Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in the upper Panels. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. Veh - vehicle.

DUSP1 activation in subconfluent and confluent endothelial cells after DHA treatment

The dual specificity phosphatase (DUSP) family is responsible for MAPK de-phosphorylation, which negatively regulates MAPK activity. To examine whether DUSP1 plays a role in the regulation of p38 MAPK by DHA, activation of DUSP1 was first investigated after DHA treatment in the two growth states. Results show that DUSP1 was activated by DHA at later time points in both subconfluent (Figure 4.5A upper blot) and confluent (Figure 4.5A middle blot) endothelial cells, but early activation of DUSP1 was only observed in confluent cells (Figure 4.5A middle blot). To examine whether DUSP1 was involved in negative regulation of p38 MAPK activation with DHA treatment, BCI (DUSP1/6 inhibitor) was added to confluent cells treated with DHA. Figure 4.5A (lower blot) shows that phosphorylation of p38 MAPK was significantly increased at 2 h in cell treated with BCI and DHA compared to cells treated with DHA (Figure 4.5B).

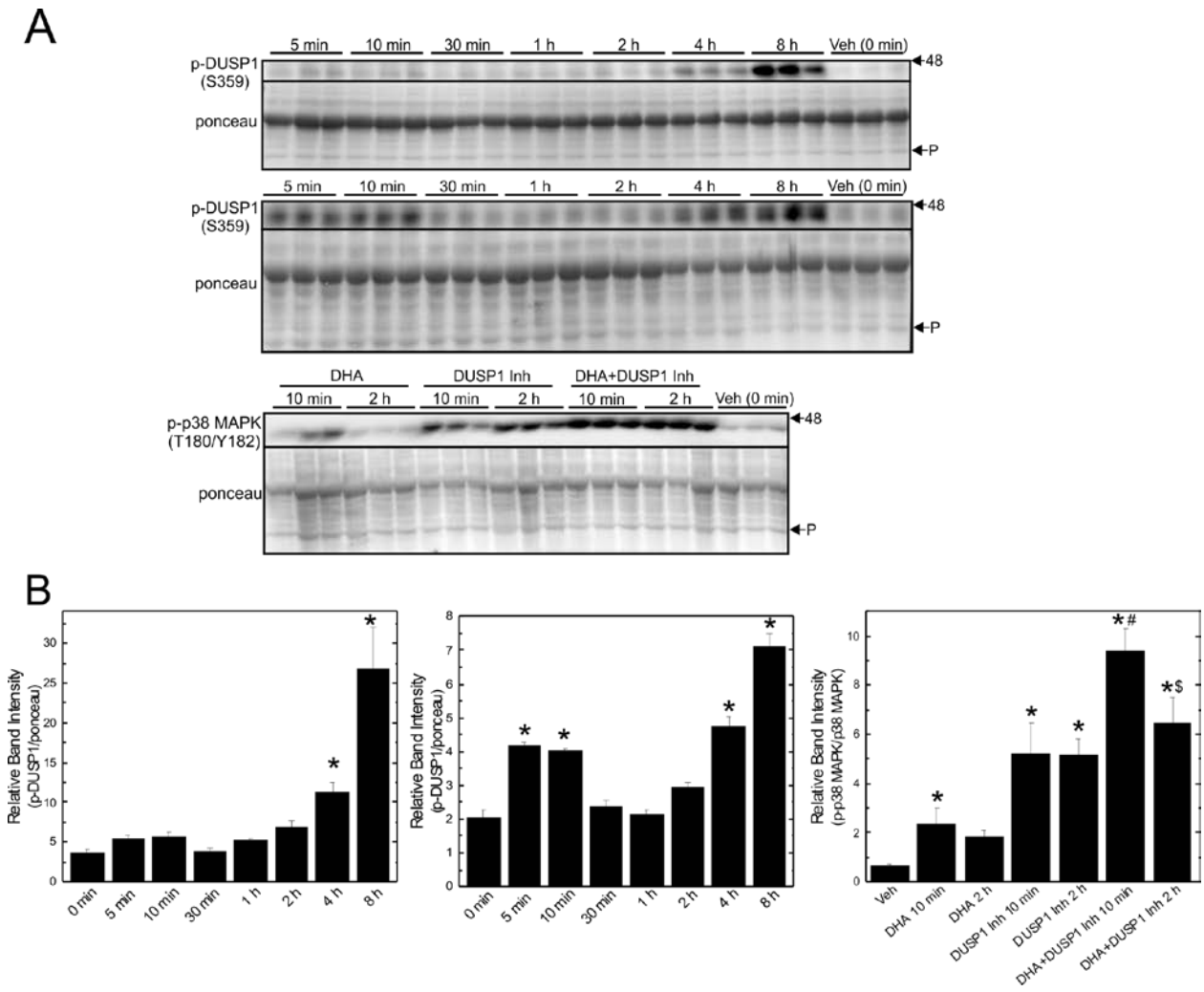
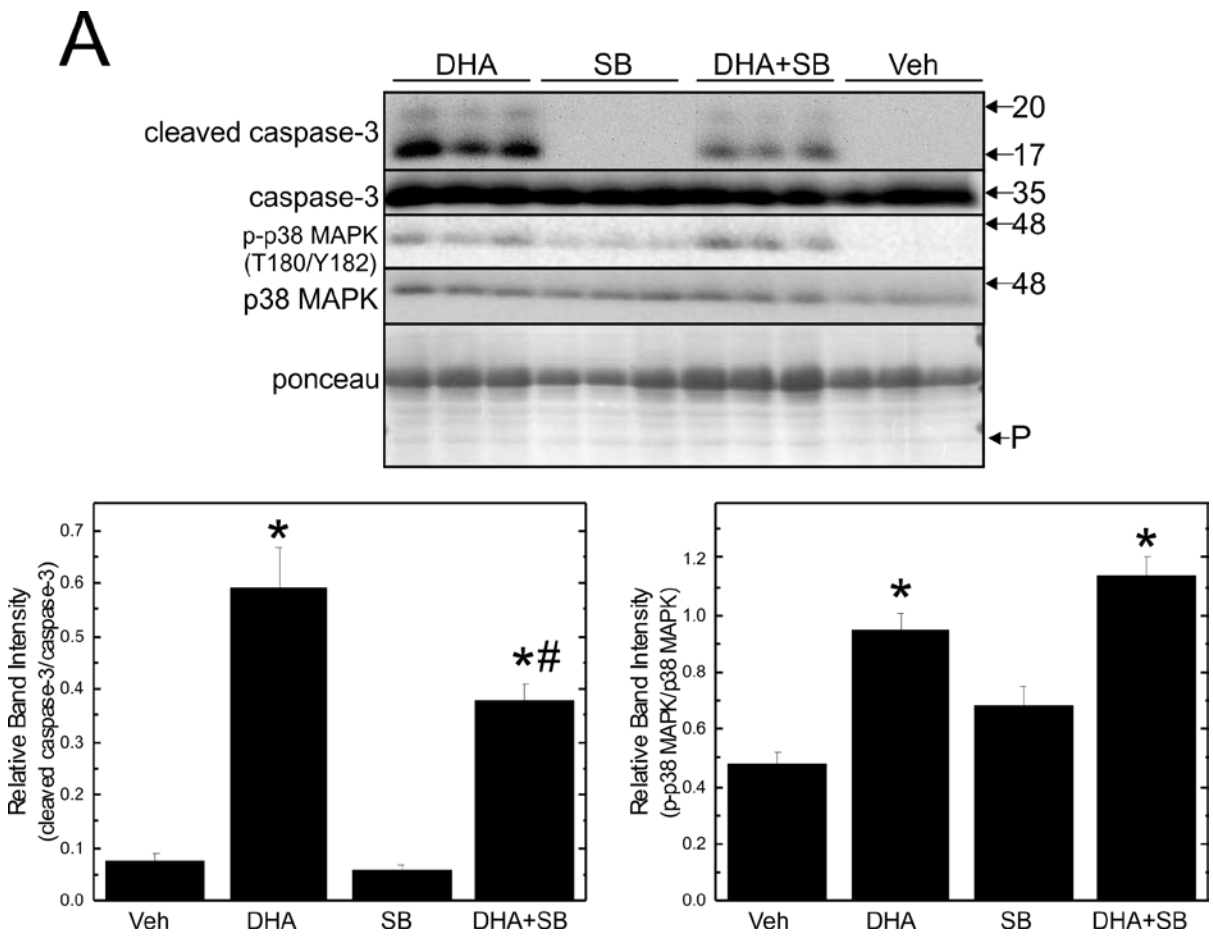

A
5 min
10 min
30 min
1 h
2 h
4 h
8 h
Veh (0 min)
p-DUSP1
(S359)
ponceau
48
P
DHA
DUSP1 Inh
DHA+DUSP1 Inh
p-p38 MAPK
(T180/Y182)
B
Relative Band Intensity
(p-DUSP1/ponceau)
Relative Band Intensity
(p-p38 MAPK/p38 MAPK)
0 min
5 min
10 min
30 min
1 h
2 h
4 h
8 h
Veh
DHA 10 min
DHA 2 h
DUSP1 Inh 10 min
DUSP1 Inh 2 h
DHA+DUSP1 Inh 10 min
DHA+DUSP1 Inh 2 h

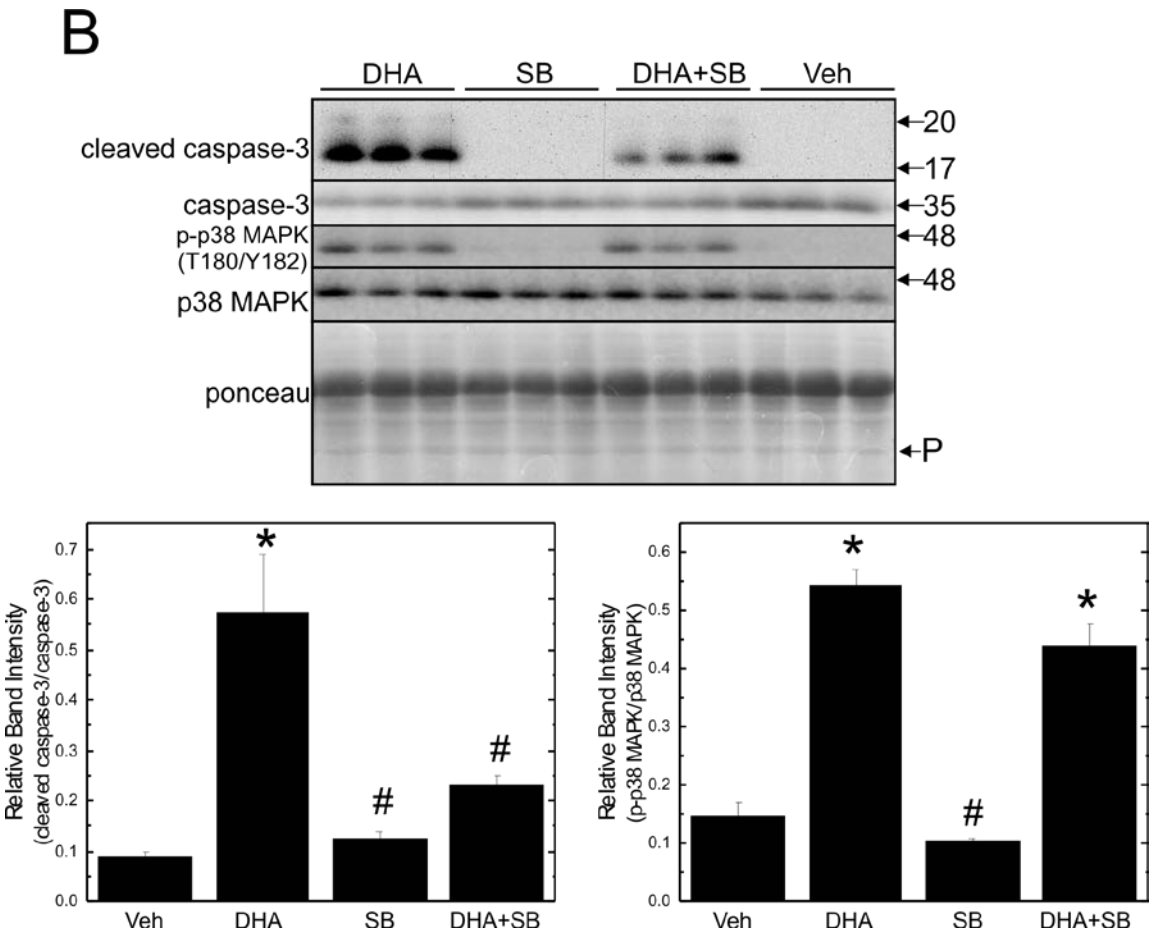
Figure 4.5 DUSP1 is activated in both subconfluent and confluent cells.

Subconfluent (Panel A upper blot) and confluent (Panel A middle blot) EA.hy926 endothelial cells were treated with 125 μM DHA for various times (0 min - 8 h). Levels of p-DUSP1 were determined by Western blotting of cell lysates prepared after treatment for the various times (0 min, 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h). Confluent EA.hy926 endothelial cells were treated with DHA (125 μM) and BCI (DUSP1 inhibitor, 5 μM) individually and in combination with DHA for 10 min and 2 h (Panel A lower blot). For the combined treatment, DUSP1 inhibitor was added to the cells 30 min before adding DHA. Levels of p-p38 MAPK were determined by Western blotting of cell lysates after each treatment. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panel A. Densitometry (Panel B) was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different from 0 min. $^{\#}$Significantly different from 10 min DHA treatment. $^{\$}$Significantly different from 2 h DHA treatment. Significance was set at $p<0.05$. Veh - vehicle.

Synergistic activation of p38 MAPK and caspase-3

To investigate the cross-talk between p38 MAPK and caspase-3, p38 MAPK inhibitor (SB202190), and caspase-3 inhibitor (Z-DEVD-FMK) were added to cells treated with DHA. When SB202190 was added to confluent cells before DHA treatment, thus before the early p38 MAPK activation occurred, caspase-3 cleavage at 8 h was significantly lower compared to DHA treatment without SB202190 (Figure 4.6A). Likewise, when SB202190 was added 2 h after DHA, thus after early activation of p38 MAPK, caspase-3 cleavage at 8 h was also significantly reduced (Figure 4.6B). These results indicated that the early activation of p38 MAPK was likely not responsible for caspase-3 activation. Rather, it appears that the minor activation of p38 MAPK seen at the 8 h time point may be responsible for the activation of caspase-3 seen at 8 h. On the other hand, when Z-DEVD-FMK was added to the cells before DHA treatment, p38 MAPK activation was significantly reduced (Figure 4.6C). Caspase-3 cleavage was not affected because Z-DEVD-FMK binds to the catalytic domain of caspae-3 thus inhibiting its catalytic activity (Figure 4.6C). This result indicates that caspase-3 may in turn up-regulate p38 MAPK.


A
DHA
SB
DHA+SB
Veh
cleaved caspase-3
caspase-3
p-p38 MAPK
(T180/Y182)
p38 MAPK
ponceau
20
17
35
48
48
P
Relative Band Intensity
(cleaved caspase-3/caspase-3)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
*
*#
Veh
DHA
SB
DHA+SB
Relative Band Intensity
(p-p38 MAPK/p38 MAPK)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
*
Veh
DHA
SB
DHA+SB


B
DHA
SB
DHA+SB
Veh
cleaved caspase-3
caspase-3
p-p38 MAPK
(T180/Y182)
p38 MAPK
ponceau
20
17
35
48
48
P
Relative Band Intensity
(cleaved caspase-3/caspase-3)
Veh
DHA
SB
DHA+SB
Relative Band Intensity
(p-p38 MAPK/p38 MAPK)
Veh
DHA
SB
DHA+SB

C

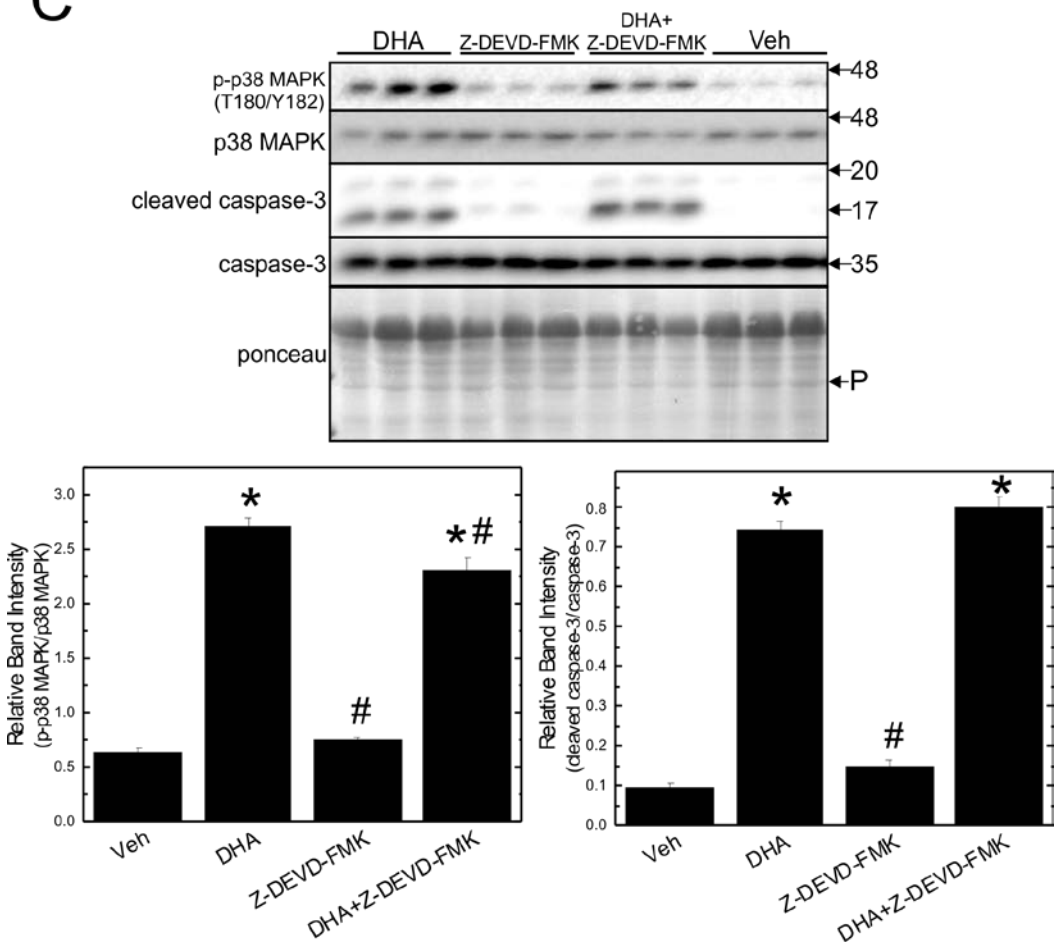

DHA
Z-DEVD-FMK
DHA+
Z-DEVD-FMK
Veh
p-p38 MAPK
(T180/Y182)
p38 MAPK
cleaved caspase-3
caspase-3
ponceau
48
48
20
17
35
P
Relative Band Intensity
(p-p38 MAPK/p38 MAPK)
Relative Band Intensity
(cleaved caspase-3/caspase-3)
Veh
DHA
Z-DEVD-FMK
DHA+Z-DEVD-FMK

Figure 4.6 Cross-talk between p38 MAPK and caspase-3.

Confluent EA.hy926 endothelial cells were treated with 125 μM DHA for 8 h. SB202190 (1 μM) was added to the cells 30 min before adding DHA (Panel A), and 2 h after adding DHA (Panel B) in the combined treatments. Z-DEVD-FMK (20 μM) was added to the cells at the same time as DHA for the combined treatment (Panel C). Levels of cleaved caspase-3, caspase-3, p-p38 MAPK and p38 MAPK were determined by Western blotting of cell lysates prepared after each treatment. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in upper parts of each Panel. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Treatment was significantly different from vehicle. $^{\#}$Treatment was significantly different from DHA treatment. Significance was set at $p<0.05$. Veh - vehicle.

MKK3/MKK6 phosphorylation occurs in parallel with p38 MAPK activation after DHA treatment

MKK3/MKK6 are the major upstream kinases that phosphorylate p38 MAPK [23]. Figure 4.7 shows that MKK3/MKK6 were activated by DHA treatment, and their activation pattern parallels that of p38 MAPK, in both subconfluent (Figure 4.7A upper blot) and confluent (Figure 4.7A lower blot) endothelial cells. This validated that MKK3/MKK6 lies in the upstream of p38 MAPK.

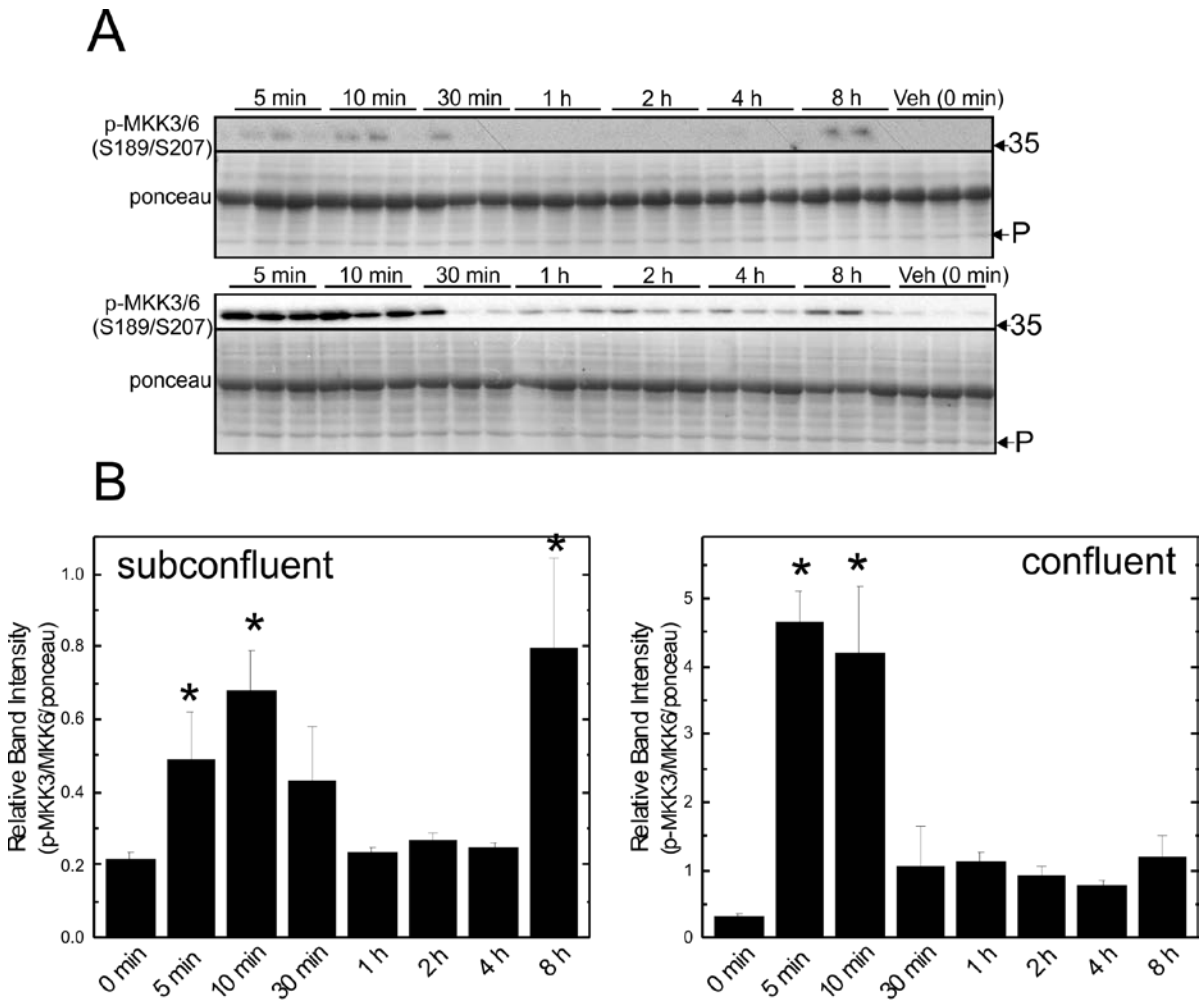

A
5 min
10 min
30 min
1 h
2 h
4 h
8 h
Veh (0 min)
p-MKK3/6
(S189/S207)
ponceau
35
P
B
subconfluent
confluent
Relative Band Intensity
(p-MKK3/MKK6/ponceau)
0 min
5 min
10 min
30 min
1 h
2h
4 h
8 h

Figure 4.7 MKK3/MKK6 activation in subconfluent and confluent cells.

Subconfluent (Panel A upper blot) and confluent (Panel A lower blot) EA.hy926 endothelial cells were treated with 125 μM DHA for various times (0 min - 8 h). Levels of p-MKK3/MKK6 were determined by Western blotting of cell lysates prepared after treatment at each time point (0 min, 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h). Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panel A. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. Veh - vehicle.

Regulation of p38 MAPK activity by caspase-3 does not involve DUSP1 or MKK3

Caspase-3 is an important mediator of apoptosis that functions as a protease to catalyze the cleavage of specific proteins in the cytoplasm. It was shown above that combined treatment of DHA and caspase-3 inhibitor significantly reduced p38 MAPK phosphorylation compared to DHA treatment (Figure 4.6C), which implies a regulatory relation between caspase-3 and p38 MAPK. However, how this regulation occurs is unknown. Since the level of p38 MAPK activity represents the equilibrium between de-phosphorylation by DUSP1 and phosphorylation by MKK3/MKK6, we examined whether the lack of p38 MAPK phosphorylation at later time points in DHA-treated confluent cells relative to DHA-treated subconfluent cells (Figure 4.3) was due to modulation of DUSP1 or MKK3 levels by proteolysis via caspase-3. Western blotting was therefore used to quantify the levels of DUSP-1 and MKK3 in confluent endothelial cells 8 h after DHA treatment. Degradation of DUSP1 and MKK3 was not observed after cells were treated with either DHA or caspase-3 inhibitor (Z-DEVD-FMK), or both combined for 8 h (Figure 4.8). The results indicated that DUSP1 or MKK3 were not involved in the regulation of caspase-3 on p38 MAPK activity.

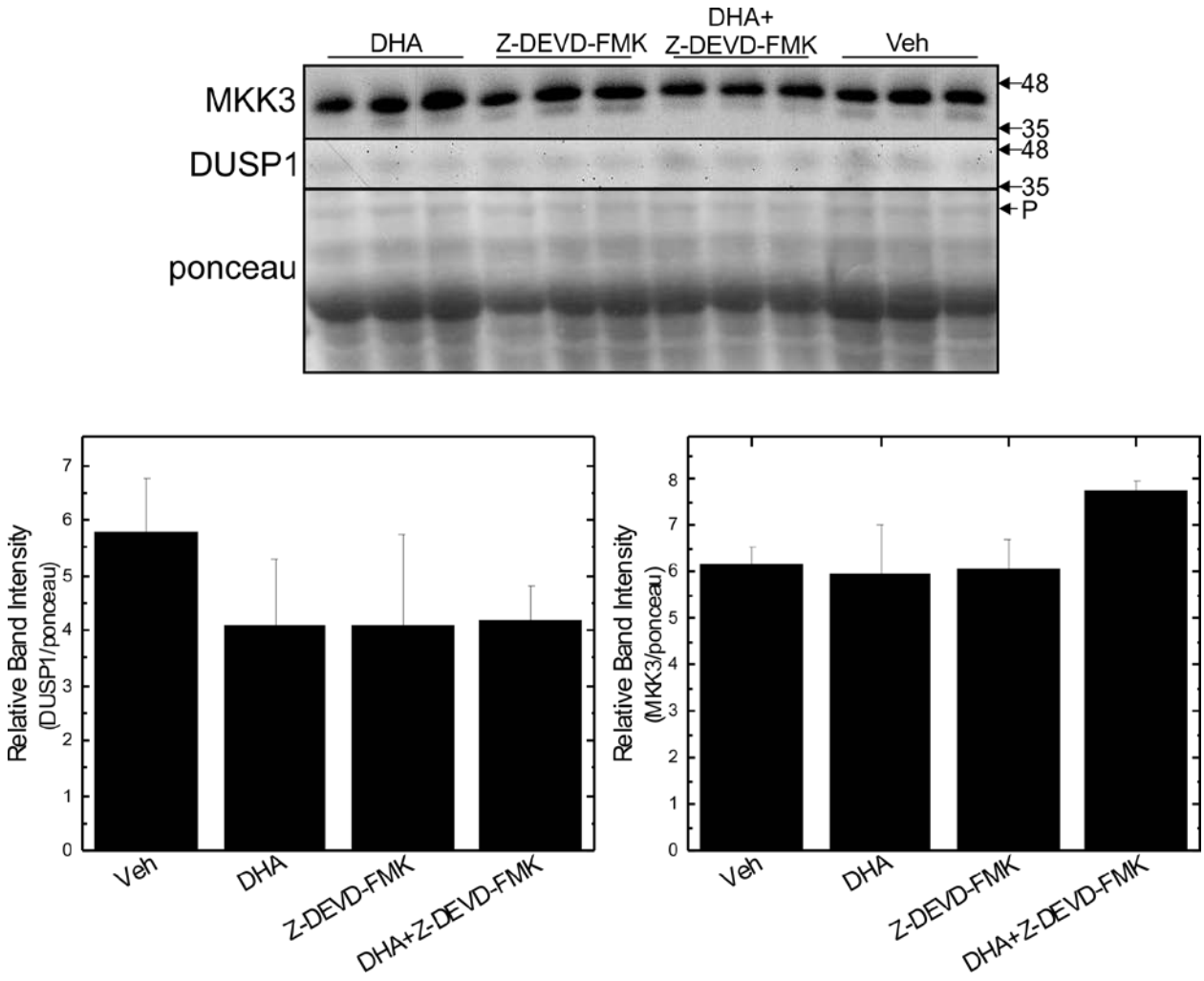

DHA
Z-DEVD-FMK
DHA+
Z-DEVD-FMK
Veh
MKK3
DUSP1
ponceau
48
35
48
35
P
Relative Band Intensity
(DUSP1/ponceau)
Relative Band Intensity
(MKK3/ponceau)
Veh
DHA
Z-DEVD-FMK
DHA+Z-DEVD-FMK

Figure 4.8 Effect of caspase-3 proteolytic activity on MKK3 and DUSP1 levels in DHA-treated confluent cells.

Confluent EA.hy926 endothelial cells were treated with DHA (125 μM) and Z-DEVD-FMK (caspase-3 inhibitor, 20 μM), individually and in combination for 8 h. In the combined treatment, Z-DEVD-FMK was added at same time as DHA. Protein levels of DUSP1 and MKK3 were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in upper Panel. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeld P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from veh. Veh - vehicle.

Inhibition of caspase-8 does not block caspase-3 activation

Activation of caspase-3 can result from stimulation of either the extrinsic pathway or mitochondria-dependent apoptotic pathway. The extrinsic pathway involves caspase-8 activation while intrinsic pathway requires activation of capase-9. To determine which pathway was involved in caspase-3 activation by DHA in confluent cells, caspase-8 inhibitor (Z-IETD-FMK) was added to cells treated with DHA. Figure 4.9 showed that the presence of the caspase-8 inhibitor in DHA-treated cells did not block caspase-3 activation, but rather increased it. Controls with DHA and inhibitor alone showed neither treatment affected caspase-3. The failure to block the activation of caspase-3 via caspase-8 inhibitor indicates that caspase-3 activation does not involve the extrinsic pathway.

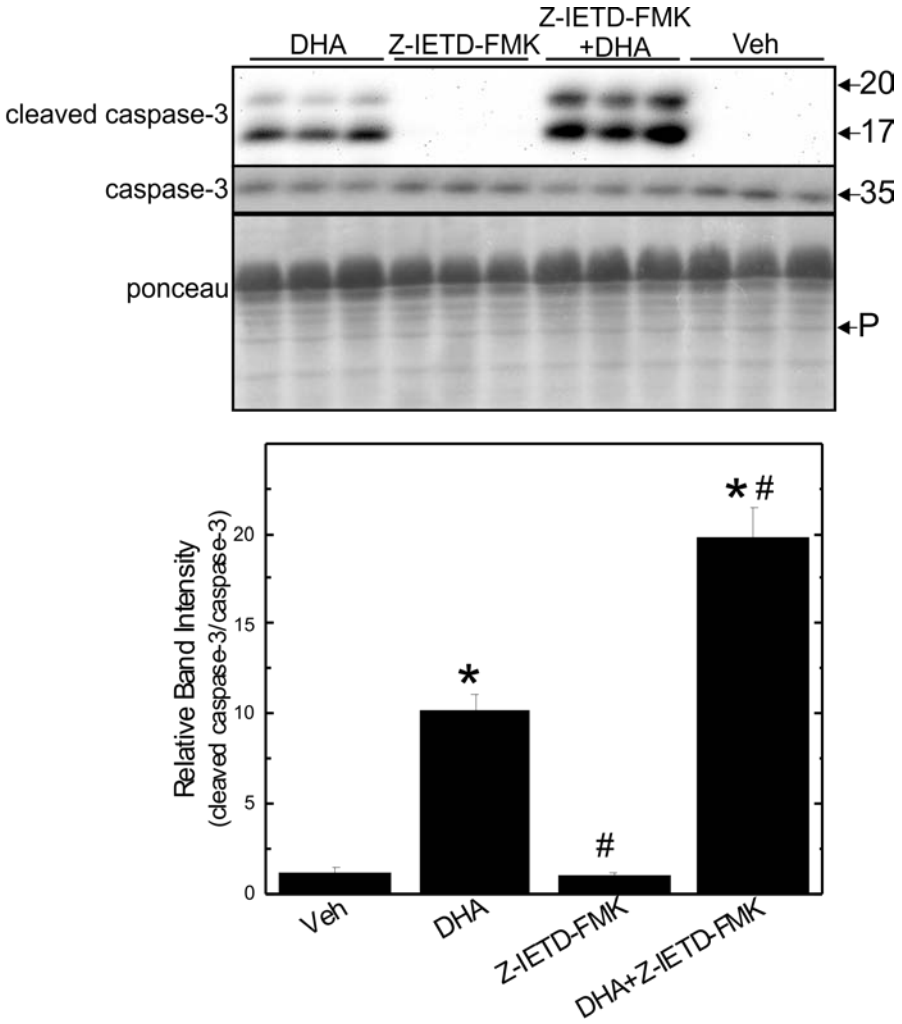


Figure 4.9 Effect of caspase-8 inhibition on caspase-3 activation.

Confluent EA.hy926 endothelial cells were simultaneously treated with DHA (125 μM) and Z-DEVD-FMK (caspase-8 inhibitor, 20 μM). Levels of cleaved caspase-3 and caspase-3 were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panel A. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Treatment was significantly different from vehicle. #Treatment was significantly different from DHA treatment. Significance was set at $p<0.05$. Veh - vehicle.

Caspase-9 is activated with DHA treatment in confluent cells

To verify that activation of capase-3 occurs through the alternative mitochondria-dependent pathway, cleavage of caspase-9 was monitored. It was found that caspase-9 was activated at 4 h by DHA treatment (Figure 4.10), which is the time at which caspase-3 activation occurs (Figure 4.2). This finding suggests that caspase-3 activation in response to DHA is dependent upon the mitochondria pathway via caspase-9.

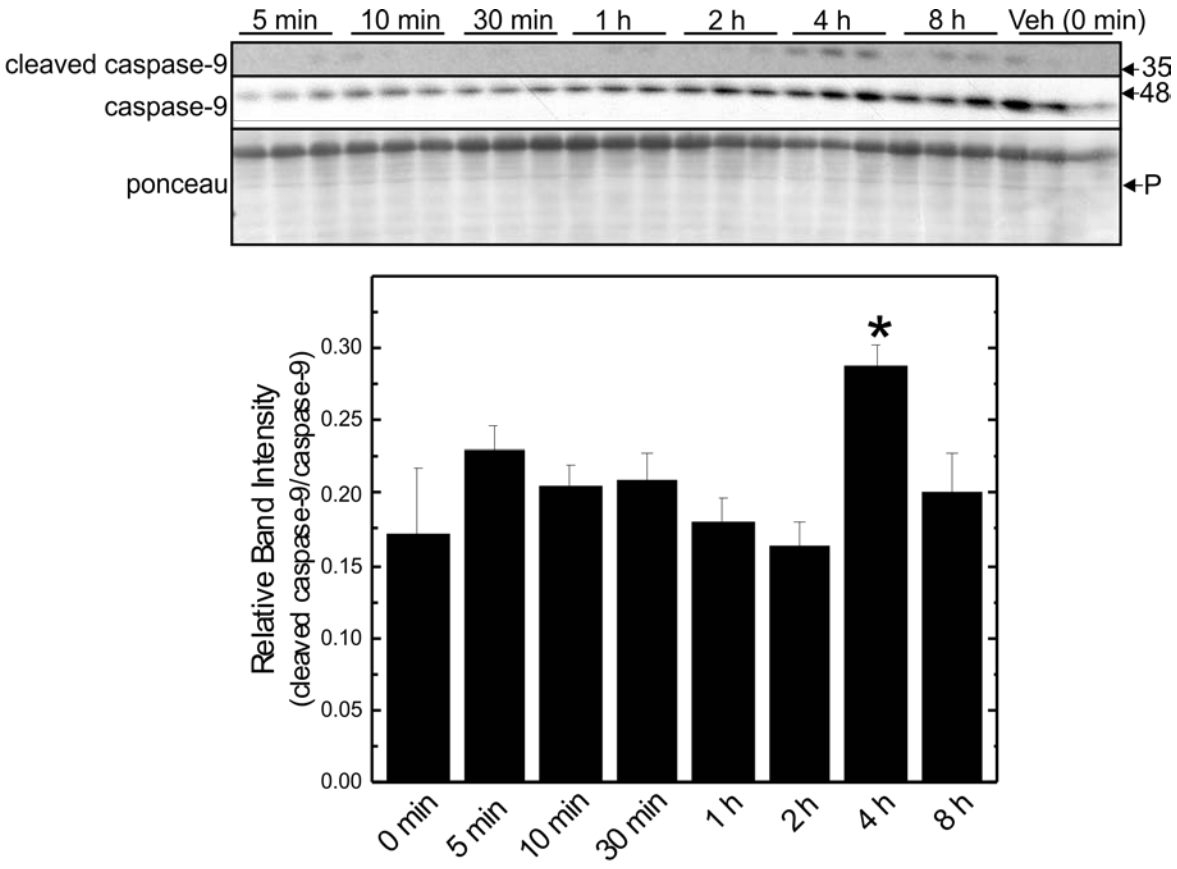


Figure 4.10 Examination of caspase-9 activation with DHA treatment of confluent cells. Confluent EA.hy926 endothelial cells were treated with 125 μM DHA for various times (0 min - 8 h). Levels of cleaved caspase-9 and caspase-9 were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panel A. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. Veh - vehicle.

4.7 Discussion

The novel finding of the present study was that DHA induces apoptosis selectively in confluent endothelial cells but not growing cells through a p38 MAPK and caspase-3 dependent pathway, in which p38 MAPK and caspase-3 form a positive regulatory loop since inhibiting either one resulted an increased activation of the other. Our studies provide evidence that DHA-induced apoptosis was via the intrinsic mitochondria-dependent pathway as activation of both caspase-9 and caspase-3 was detected. MKK3/MKK6 and DUSP1 were also shown to respond to DHA, thus providing the equilibrium necessary for the regulation of p38 MAPK activity by DHA treatment. Our study is the first to report that activation of MAPKs (p38, ERK1/2 and JNK) was not only growth-dependent, but also followed a specific temporal sequence in EA.hy926 endothelial cells in response to various PUFAs. Only p38 MAPK was induced by DHA at the early time points in confluent cells, whereas DHA induced activation of p38, ERK1/2 and JNK at later time points in subconfluent cells, and at both early and later time points in confluent cells. The activation pattern of p38 MAPK by DHA was unique since other PUFAs (LA, AA, ALA and EPA) only caused early time point activation of p38 MAPK. The activation pattern of MAPKs in growing and confluent cells reveals a complex interplay between these kinases that has not been reported previously.

We observed DHA-induced apoptosis in confluent but not subconfluent EA.hy926 endothelial cells when treated with 125 μM DHA. In support of our results, Krieglsterin et al. [32] showed 200-400 μM DHA induced apoptosis in 90% confluent HUVEC, but they did not study the response of subconfluent cells. On the contrary, Kim et al. [33] found 40 μM DHA induced apoptosis in proliferating but not confluent HUVEC, and Schaefer et al. [34] found 10 μM DHA did not induce HUVEC apoptosis after 6 h. Factors such as concentration and treatment time, culture conditions, growth state, and difference in cell line or origin of

endothelial cells could be reasons for the inconsistency in results. The present study used EA.hy926 cells which are a cell line of HUVEC origin [35] whereas Krieglsterin et al. [32] and Kim et al. [33] used primary HUVECs and lower concentration of DHA. Artwohl et al. [15] found high concentrations (300 μM) of LA, AA and ALA caused apoptosis of human aortic endothelial cells and endothelial progenitor cells but not human retinal endothelial cells. It is also unknown whether and how the basement membrane could mediate the response of endothelial cells to DHA or other dietary factors. Previously, we reported the importance of growth state as well as the substrate in the endothelial cell response to fatty acids [19]. It is possible that the Matrigel substrate we used influences the intracellular actions of DHA. In part, this may result from the activation of additional pathways that are required for the full biological effects of DHA leading to apoptosis, such as phosphorylation of p53, disruption of mitochondria membrane potential and increased production of ROS [33]. Examining the interplay amongst these various intracellular mediators will be necessary to fully understand how apoptosis is regulated in these cells. Kim et al. [33] study also emphasized the role of growth state. Our previous results showed that growing cell viability was significantly reduced by 125 μM DHA, and that cells lose adherence [19]. Therefore, the impact of DHA on growing EA.hy926 cells might be though a different mechanism, which warrants further investigation.

This study is the first to report that direct MAPK pathway (p38, ERK1/2, JNK) activation occurs in human endothelial cells by DHA, which is unlike studies that have reported the modulation of MAPK signaling by DHA in TNF-α treated endothelial cells [36, 37]. We had previously reported that p38 MAPK activity was elevated up to 8 h with DHA treatment in both subconfluent and confluent states, and showed that activation pattern was distinct. The current study extends this to a comparison of all three MAPKs and shows that the DHA-induced activation of the MAPKs was growth state-dependent. In subconfluent cells, only p38 was

activated at early time points (5 and 10 min) by DHA whereas p38, ERK1/2 and JNK were induced at later times (4 and 8 h). In confluent cells, DHA induced early time point activation of p38, ERK1/2 and JNK, but only ERK1/2 and JNK were induced at the later time point activation. Only one previous study performed by Kim et al. [33] has shown that 40 μM DHA resulted in p38 MAPK activation at 5-30 min in proliferating HUVEC, and increased p38 MAPK activation after 15-60 min in confluent cells. These results are consistent with ours in that DHA induced early activation of p38 MAPK in both growing and confluent endothelial cells, however, Kim et al. [33] did not examine p38 MAPK for a longer period of time, nor did they examine the activation of ERK1/2 and JNK at same time.

Like DHA, direct activation of MAPK in endothelial cells by other PUFA (LA, AA, ALA and EPA) has received limited attention. Our study is the first to report the differential modulation of MAPK activity in growing endothelial cells by different PUFAs. We observed that LA, AA, ALA and EPA only caused early time point activation of p38 MAPK. Hii et al. [38] were the first to report PUFA-dependent activation of p38 MAPK as indicated by the fact that 20 μM AA induced phosphorylation of p38 MAPK after 4 min in HUVEC. Hennig et al. [39] showed 90 μM LA activated p38 MAPK in confluent porcine pulmonary artery endothelial cells at 10 min and returned to baseline after 30 min within the 6 h observation period. Zheng et al. [40] reported that 90 μM LA induced activation of p38 MAPK and ERK in confluent EA.hy926 endothelial cells, with maximum phosphorylation at 5 min and 10 min, respectively (treatment up to 20 min). Evans et al. [41] showed p38 MAPK activation peaked at 2 h with 50 μM AA and lasted up to 3 h in in primary human brain endothelial cells, while LA did not activate p38 MAPK at the same concentration. Zgheel et al. [42] showed EPA and DHA at a ratio of 6:1 caused phosphorylation of p38 MAPK, ERK1/2 and JNK after 30 min in porcine coronary artery endothelial cells. Based on these observations, it is clear that MAPKs play

important roles in mediating the cellular effects of these PUFA in endothelial cells, but more studies are required to elucidate the cellular events modulated by MAPKs.

We observed activation of p38 MAPK, ERK1/2 and JNK over a period of 8 h in a temporal sequence, with different patterns according to the growth state of the cells. The consequences of temporal activation may be the need to activate different downstream targets at different times, which are required to determine the overall cellular response. MAPK cross-talk is a concept that has been supported by many studies. Previously, Xie et al. [43] showed that the relative activities of JNK and p38 compared to ERK1/2 and big MAPK may be important to determine apoptosis in rat PC-12 pheochromocytoma cells. Other studies have also shown the ability of MAPKs to regulate the activity of each other. Paumelle et al. [44] showed ERK1/2 regulated expression of MAPK phosphatase 2 that deactivated JNK, and other groups showed p38 downregulated ERK1/2 [45-47].

Our data indicate a positive regulatory loop between p38 MAPK and casapse-3. It is known that p38 MAPK acts an upstream regulator of caspase-3 in endothelial cells undergoing apoptosis [48]. This viewpoint is supported in our study, since inhibition of p38 MAPK results in a decline in caspase-3 activation. We also showed that capase-3 influences p38 MAPK as caspase-3 inhibition led to a reduction of p38 MAPK activation. It is noteworthy that even though the activation of p38 MAPK at later time points by DHA in confluent cells was weak and not statistically significant, downstream targets of p38 MAPK might still be able to carry the signals and induce caspase-3 activation. Cross-talk between caspase-3 and p38 MAPK was seen previously in human cerebral microvascular endothelial cells in response to hypoxia-reoxygeneration [49], as well as in human aortic endothelial cells where apoptosis was induced by a high concentration of D-glucose [50]. Additionally, Lee et al. [49] and Nakagami et al. [50] found caspase-3 cleaves MAPK kinase kinase 1 (MEKK1), the kinase that is upstream of

MKK3/MKK6 and p38 MAPK. Although we did not examine the contribution of MEKK1, we showed that caspase-3 did not regulation p38 MAPK through kinase MKK3/MKK6.

The p38 MAPK pathway regulates important cellular responses related to cellular stress and inflammation. Chronic inflammation leads to endothelial cell dysfunction and vascular disease, thus both the degree and duration of p38 MAPK activation has to be tightly regulated. DUSP, which is a family of 10 phosphatases, differ in expression, localization and substrate specificity [51]. DUSP1 was originally found to dephosphorylate and inactivate ERK1/2, but was also shown later to target p38 and JNK [27]. In our study, we showed inhibition of DUSP1 increased p38 MAPK activity, which implied that DUSP1 was involved in negative regulation of p38 MAPK phosphorylation. However, DUSP1 activation was not concurrent with p38 MAPK activation or inactivation in either growing or confluent cells suggesting DUSP1 performs a different regulatory function in growing and confluent endothelial cells.

4.8 Conclusions

Our results suggest that growth state plays important role in mediating endothelial cell response to DHA. An illustrative figure can be viewed in Figure 4.11. First, DHA-induced apoptosis in confluent cells but not growing cells, and it is a process that requires p38 MAPK and mitochondria-dependent activation of caspase-3. Although the death pathway in growing cells by DHA was not further examined, activation of MAPKs may play a role. Second, we have observed a growth state-dependent activation of MAPKs (p38, ERK1/2, JNK), which also follows a temporal sequence, in response to DHA treatment. This may reveal a complex interplay between these kinases. Regardless of the mechanisms involved, the novel findings of our study suggest that precautions may be warranted when DHA supplements are taken in high amounts. The concentration of DHA (125 μM) used in this study is within physiological range; Conquer et al. [52] detected up to 400 μM DHA in phospholipid after consuming 1.5 g DHA per

day for 6 weeks. Endothelial cells can be exposed to excessively high concentration of fatty acids including DHA due to the lipolysis of lipoprotein occurring at the surface of endothelium as well as free fatty acids released into the circulation from lipolysis of internal triglycerides via hormone sensitive lipase.

An important implication of our results is that DHA may be able to influence endothelial cell function when the endothelium is healthy and when blood vessels are undergoing repair after a vascular trauma, and that the underlying mechanisms in each condition are different. Based on our current understanding, it may be postulated that DHA may have an adverse effect on proliferating endothelial cells while induction of apoptosis in confluent but not growing cells may be beneficial to the vascular system. Unlike necrosis, when a cell ruptures, releases cellular content and induces inflammation, apoptosis is a type of cell death that does not initiate inflammation because under normal conditions, apoptotic cells do not rupture but form apoptotic bodies that are removed from the site by phagocytic cells. This would be beneficial when the endothelium is undergoing repair due to trauma or other causes. However, this conclusion needs further study with respect to the pertinent functions of DHA and endothelial cells. Addressing these aspects will require further investigation in a more physiological model.

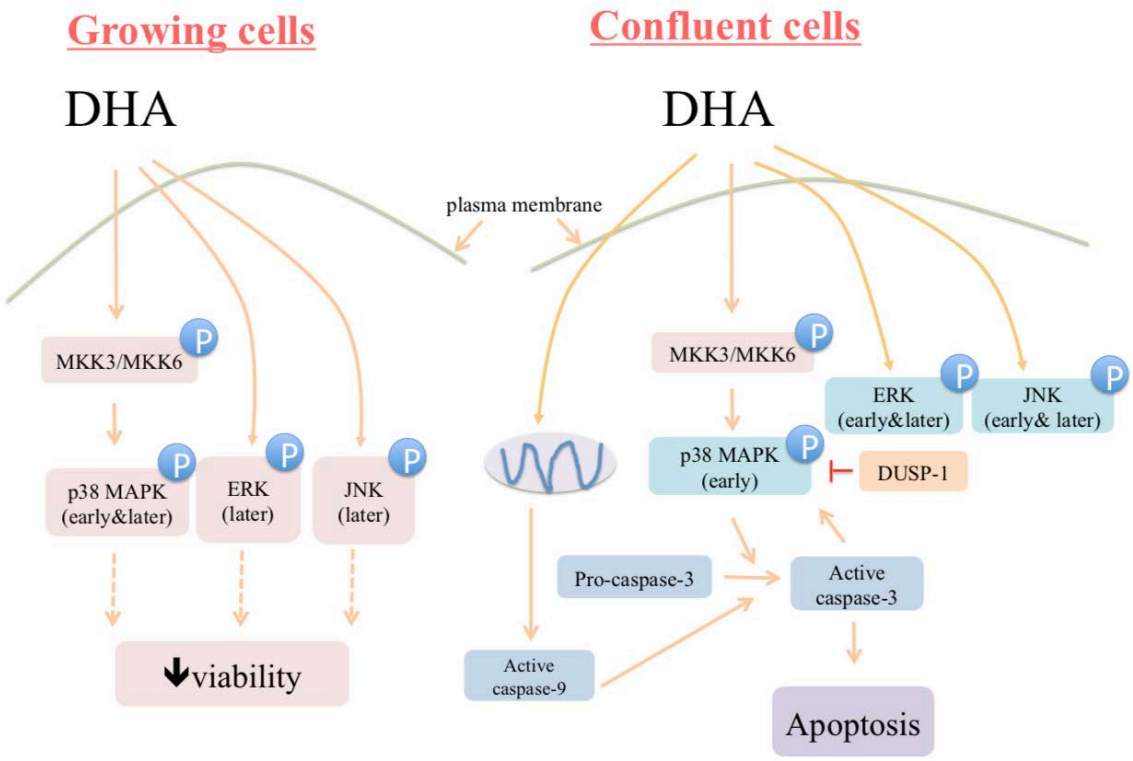


Figure 4.11 A summary of the effects of DHA on MAPK and caspase signaling with respect to cell viability in growing and confluent EA.hy926 endothelial cells.

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Chapter 5: PD146176 Modulates Human Endothelial Cell Viability and Proliferation Via a p38 MAPK-Akt-PPARα Dependent Pathway: Potential Role of CYP-derived Oxylipins

5.1 Contribution Statement

I, Youjia Du, made the following contribution to this study:

- Developed the study concept and experimental design with other contributors
- Conducted all experiments and data analysis
- Wrote the manuscript
- Interpreted results and edited the manuscript with other contributors

Other contributors:

- Peter Zahradka
- Carla G. Taylor
- Harold M. Aukema

5.2 Study Rationale

The results shown in the previous chapter have reinforced the importance of growth state (subconfluent, confluent) in the EA.hy926 endothelial cell response to DHA. It was not only shown that DHA-induced apoptosis in EA.hy926 endothelial cells is a growth state-dependent process, but also suggested that apoptosis in confluent cells requires activation of caspase-3 and MAPKs (especially p38 MAPK). PUFAs are precursors of lipid mediators such as oxylipins, which have been shown to influence all aspects of endothelial cell function. However, due to the large number of oxylipin species and their complex interplay, this emerging field still requires more studies before we will completely understand their biological actions as individual oxylipins. At same time, studies examining the interactions between oxylipins and EA.hy926 cells are limited. Adding to the complexity of the analysis is the fact oxylipins are generated via three major families of oxylipin-producing enzymes, COX (COX1, COX2), CYP (numerous isoforms) and LOX (5-LOX, 12-LOX, 15-LOX). To date, studies examining the effects of the LOX isoforms on endothelial cell function have produced inconsistent results, and the effects of the LOX products are largely unknown. Since inhibition of the COX pathway led to development of non-steroidal anti-inflammatory drugs (NSAIDs) and therapeutics for managing inflammation, the contribution of the LOX pathway to endothelial cell function is also worth investigating. Preliminary experiments showed that inhibition of COX with suprofen (25 μM) and lornoxicam (0.1 μM) or inhibition of 5-LOX with NDGA (5 μM) did not produce a change in BrdU incorporation (Appendix Figure A.2); however, there was a dose-dependent inhibition with a presumed 5-LOX inhibitor - NDGA (Appendix Figure A.1). Combined treatment of AA/DHA and NDGA produced the greatest reduction in cell proliferation compared to fatty acid treatment alone (Figure A.2). Therefore, the focus of the experiments described in this chapter was on LOX pathways, and to investigate the role of LOX on EA.hy926 cell viability and

proliferation. While a limited number of studies examining this question have been reported in the literature, knowledge of how the LOX pathways contribute to human endothelial cell physiology is still largely unknown. Therefore, this chapter presents experiments that were designed to address the role of LOX in modulating endothelial cell function by inhibiting 5-, 12-, and 15-LOX pathways individually and in combination using specific inhibitors and examining EA.hy926 endothelial cell viability and proliferation. The study also investigated the changes in the profile of oxylipin production with 15-LOX inhibition (as only use of the presumed15-LOX inhibitor was shown to have an effect) to not only verify the specificity of the inhibitor but also identify oxylipins that may be associated with cell viability and proliferation.

5.3 Abstract

Endothelial cells line the inner surface of blood vessels, and play important roles in maintaining vascular homeostasis. Oxylipins are oxygenated derivatives of PUFAs generated by three enzymatic pathways, COX, LOX and CYP. Oxylipins function as lipid mediators that regulate various aspects of endothelial cell physiology, including apoptosis, proliferation, angiogenesis, migration, and barrier function, and have been implicated in the pathogenesis of vascular disease. However, the consequences of LOX inhibition in endothelial cells have not received much attention. The current study examined whether LOX pathways play a role in modulating viability and proliferation of EA.hy926 endothelial cells in the confluent and growing states. Potential molecular mechanisms and cross-talk between the signaling pathways were studied using inhibitors, while oxylipins were profiled with LC/MS/MS. Our study showed that PD146176, which is commonly used as a 15-LOX inhibitor, negatively affected the viability and proliferation of growing cells, and reduced the viability of confluent cells, possibly through an up-regulation of p38 MAPK and PPARα and down-regulation of Akt. We also observed that PPARα had a stimulatory effect on Akt, while p38 MAPK inhibited PPARα. PD146176 did not inhibit 15-LOX in EA.hy926 cells, since products of this enzyme did not decrease. Instead, CYP products such as 5,6-, 8,9-, 11,12-EpETrE, 14,15-dihydroxy-eicosatrienoic acid (DiHETrE) and 12,13-dihydroxy-octadecenoic acid (diHOME) were reduced 30 min after treatment, and accumulation of 9,10,13-trihydroxy-octadecenoic acid (triHOME) occurred at both 30 min and 8 h. Consequently, it would appear that PD146176 does not function as a 15-LOX inhibitor in EA.hy926 endothelial cells. However, PD146176 negatively affects endothelial cell viability and proliferation through the modulation of p38 MAPK, PPARα and Akt signaling, potentially by modulating CYP-derived oxylipins.

5.4 Introduction

Endothelial cells form a single-cell layer called the endothelium that covers the luminal surface of all blood vessels and play important roles in the maintenance of vascular homeostasis [1]. Because of their unique location, endothelial cells act as a barrier that separates the blood and underlying tissues, yet they have selective permeability that permits regulation of molecule transport. Endothelial cells are also involved in the regulation of many vital physiological processes such as vascular tone, coagulation, and inflammation. [1-7]. Healthy endothelial cells secrete factors such as NO and PGI_2 that mediate vascular relaxation, and vasoconstrictors such as endothelin-1 that act on VSMCs to regulate vascular tone and blood flow. NO and PGI_2 are also anticoagulant factors, which prevent platelet aggregation. Endothelial cells produce t-PA and urokinase that mediate fibrinolysis, and they participate in inflammatory responses by expressing adhesion molecule and secreting cytokines to recruit immune cells.

Endothelial cells are also sensitive to a diverse variety of physical, chemical and mechanical stimuli that can cause transient cell activation, which can disturb vascular homeostasis [8]. PUFAs have been shown to influence endothelial cells in different ways [9-24]. Generally, it is believed that n-3 PUFAs (ALA, C18:3 n-3, EPA, C20:5 n-3, DHA, C22:6 n-3) are more beneficial to the cardiovascular system than n-6 PUFAs (LA, C18:2n6, AA, C20:4n6). The mechanisms by which n-3 PUFAs produce these beneficial effects involve in part the modulation of endothelial cell functions including regulation of vascular tone [25-28], inflammation [29-33], and oxidative stress [34]. More recently, PUFAs have been shown to exert their influence through the production of oxygenated products called oxylipins, which is supported by evidence that the oxylipin profile changes after various PUFA treatments [35-43], and that individual oxylipins can directly modulate endothelial cell function [44, 45]. Oxylipins can be produced through three enzymatic pathways, COX, LOX and CYP. Among them, LOX is

a non-heme, non-sulfur iron containing dioxygenase that catalyzes the insertion of oxygen into PUFAs and produces conjugated hydroperoxides [46]. Specifically, LOX adds a hydroperoxy group at carbons 5, 12 or 15 from the carboxyl end of 20-carbon fatty acids, and thus they are named 5-, 12- or 15-LOX [47]. The human genome has six LOX genes that encode six isoforms [48].

While the impact of some individual oxylipins produced by LOX on endothelial cells has been examined, most others are largely unstudied. Due to the large number of oxylipins and their complex interplay [49, 50], studying the effect of individual oxylipins is as important as looking at changes in the entire oxylipin profile. The benefits observed with COX inhibition in suppressing inflammation led to the development of NSAIDs such as aspirin and ibuprofen that function by inhibiting the production of pro-inflammatory oxylipins such as prostaglandins. The only drug approved on the market to selectively inhibit 5-LOX production of leukotrienes is Zileuton, which is used in the treatment of asthma. However, the consequence of inhibiting LOX pathways in endothelial cells is not well known. For instance, inhibiting 12/15-LOX by baicalein or knocking down 12/15-LOX in human retinal endothelial cells significantly reduced the high glucose-induced ICAM-1 expression and $CD14^+$ monocyte adhesion [51]. Studies on LOX inhibitors and LOX inhibition have been slow due to the lack of information on the specific structures of LOX as well as the presence of LOX isoforms [52].

The current study was designed to investigate the impact of LOX inhibition on endothelial cells with specific attention on cell viability and proliferation. The study hypothesized that 15-LOX inhibition, but not 5- or 12-LOX inhibition, negatively affects EA.hy926 endothelial cell viability and proliferation in a growth state-dependent manner. LOX inhibition was evaluated by determining changes in the oxylipin profile. The molecular mechanisms of LOX inhibitors were studied by Western blotting of p38 MAPK, Akt, and PPARα. p38 MAPK belongs to the serine-

threonine protein kinase family that responds to cellular stresses, pro-inflammatory cytokines and mitogen signals and regulates cell physiology including cell survival and cell cycle control by transducing signals from extracellular space to the nucleus [53]. Akt is a key mediator in cell survival and proliferation; it promotes both cell cycle progression and cell survival by inhibiting proteins such as caspase and BAD protein that are involved in apoptosis [54]. PPARs are a family of ligand-activated transcriptions factors, which include PPARα, PPARβ/δ and PPARγ. The major function of PPARs is in energy homeostasis, fatty acid and glucose metabolism, and insulin sensitization [55]. PPARs are able to regulate CYP gene expression including CYP4A classes, CYP27A1, CYP8B1, CYP7B1, etc. [56]. Some CYP products including 11,12- and 14,15-DiHETrE [57], COX and LOX products such as prostaglandins, leukotrienes, HETEs and HODEs are also ligands of PPARα [58].

5.5 Materials and Methods

Cell culture

EA.hy926 endothelial cells were purchased from ATCC (cat. CRL 2922, Manassas, Virginia, USA) and propagated as described previously on Matrigel-coated plates for up to 8 days [59]. EA.hy926 endothelial cells were seeded at a density of 9000 cells/cm^2. Cells were seeded on day 0. Subconfluent cells were used at day 4, and confluent cells were used at day 8. Cells were used within 20 passages.

Cell treatments

Three different LOX inhibitors were employed: PD146176 (6, 11-dihydro-5-thia-11-aza-benzo[a]-fluorene, Santa Cruz #200678), NDGA (nordihydroguaiaretic acid, Calbiochem #479975), and CDC (cinnamyl-3,4-dihydroxy-α-cyanocinnamate, abcam#141560). Each was dissolved in DMSO and used at concentrations from 1-100 μM to treat subconfluent and

confluent cells for up to 24 h. SB202190 (p38 MAPK inhibitor,1 μM) and GW6471 (PPARα antagonist, 2.5 μM), prepared in DMSO, were added to cells 30 min before adding PD146176.

Cell viability

Cell viability (total cell number) was assessed with the CCK-8 (cell counting kit, Dojindo Molec Tech) assay. CCK reagent was added to the cells and incubated for 90 min. The absorbance of the media was measured at 450 nm using a microplate reader.

Cell proliferation

Proliferation of cells was measured with the BrdU Cell Proliferation kit (Millipore). BrdU reagent was added to the cells and incubated for 4 h. Cells were fixed, DNA was denatured, and incorporated BrdU was detected immunochemically according to the kit instructions. Plates were measured at a dual wavelength of 450/550 nm.

Western blotting

Proteins extracted from subconfluent and confluent EA.hy926 cells were analyzed by Western blotting as previously described [59]. Primary antibodies were diluted in TBST (Tris-buffered saline with Tween-20; 150 mM NaCl, 20 mM Tris pH 7.4, 0.05% Tween 20) containing 3% BSA. The primary antibodies employed were rabbit anti-phospho-Akt (Ser473) (1:1000, Cell Signaling, #9271), rabbit anti-Akt (1:1000, Cell Signaling, #9272), rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (1:1000, Cell Signaling, #9211), rabbit anti-p38 MPAK (1:1000, Cell Signaling, #9212), rabbit anti-phospho-PPARα (Ser12) (1:1000, Abcam, #3484). HRP conjugated secondary antibodies were used at a dilution of 1:10000 in TBST containing 1% BSA. The relative band intensities were captured using a FluorChem® Q imager and quantified with AlphaView software (Alpha Innotech). The Western blot data were normalized to a Ponceau S stained band on the blot.

Oxylipin analysis

Three mL of media were collected from subconfluent cells after PD 146176 treatment, and the media was used for analysis of oxylipins. The cells were lysed 2× sample buffer (125 mM Tris-HCl pH 6.8, 2% Sodium dodecyl sulphate, 20% glycerol) and protein concentration was determined by BCA protein assay. This allowed calculation of the concentration of oxylipins produced by the cells (on a per mg protein basis) and present in the collected media. Methanol (450 μL) and 18.75 μL of antioxidant cocktail (0.2 g/L butylated hydroxytoluene, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in methanol:ethanol:H_2O [2:1:1, by vol]). were added to the samples, followed by adjusting pH to less than 3. Solid phase extraction (SPE) was performed with Strate-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water. Samples were loaded onto the columns, rinsed with 10% methanol, and eluted with methanol. Evaporated samples were resuspended in solvent A (H_2O:acetonitrile:formic acid (70:30:0.02 v/v/v)) and then subjected to analysis by HPLC-MS/MS (API 4000, AB Sciex, Canada). Quantification limits of oxylipins were set at 5 times the level above background. Oxylipins were determined using the stable isotope dilution method and expressed as picomole per mg of protein.

Statistical Analysis

One-way ANOVA, followed by Tukey's test or Dunnett's test were used to assess statistical significance, which was set at $p<0.05$ (SPSS version 20). All experiments were independently repeated at least 3 times. Within each experiment, the plotted values were derived from treatments done in triplicate (n=3) and are expressed as mean ± SEM.

5.6 Results

Effects of LOX inhibitors on cell viability and proliferation

To test whether products of a specific LOX have an effect on endothelial cell viability and proliferation, growing and confluent endothelial cells were treated with NDGA (5-LOX inhibitor) [60], CDC (12-LOX inhibitor) [61] and/or PD146176 (15-LOX inhibitor) [62] at concentrations 10× the IC_{50} for 24 h, after which cell viability and cell proliferation were assayed. In growing cells, the 15-LOX inhibitor (PD146176) reduced endothelial viability compared to DMSO (vehicle control), while neither the 5-LOX inhibitor (NDGA) nor the 12-LOX inhibitor (CDC) affected viability (Figure 5.1A). The combination of the 15-LOX inhibitor (PD146176) and the 12-LOX inhibitor (CDC) reduced the viability of growing cells to about the same degree as 15-LOX inhibitor alone (Figure 5.1A). Interestingly, combining the 15-LOX (PD146176) and 5-LOX inhibitors (NDGA) or all three inhibitors had no effect on cell viability (Figure 5.1A). Cell proliferation was affected similarly as cell viability, with the 15-LOX inhibitor (PD146176) alone reducing proliferation and combinations of the 15-LOX inhibitor (PD146176) with either NDGA or CDC or both together reducing cell proliferation to the same degree as PD146176 treatment alone (Figure 5.1B). In contrast, the 5-LOX (NDGA) and 12-LOX inhibitors (CDC) had no effect on cell proliferation (Figure 5.1B). In confluent cells, the 15-LOX inhibitor (PD146176), but not the 5-LOX inhibitor (NDGA) or the 12-LOX inhibitor (CDC), reduced cell viability (Figure 5.1C). Combination of the 15-LOX inhibitor (PD146176) with 12-LOX inhibitor (CDC) also reduced cell viability, but this effect was not seen with the combination of 5-LOX inhibitor (NDGA) and 12-LOX inhibitor (CDC) or the combination of 5-LOX inhibitor (NDGA) and 15-LOX inhibitor (PD146176) (Figure 5.1C). With regards to proliferation, no effect was seen with either the inhibitors alone or in combination (Figure 5.1D).

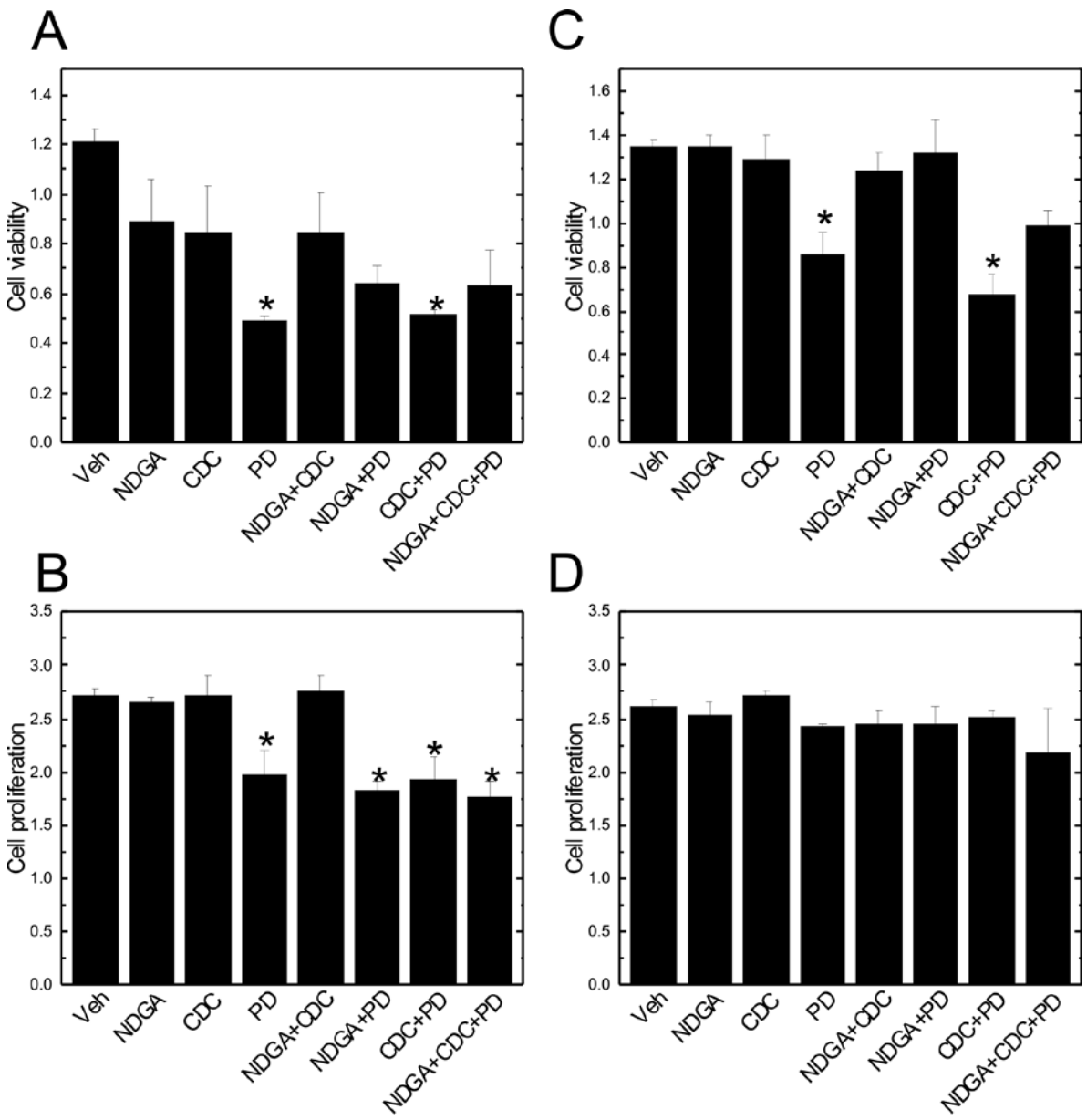

A
Cell viability
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Veh
NDGA
CDC
PD
NDGA+CDC
NDGA+PD
CDC+PD
NDGA+CDC+PD
C
Cell viability
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Veh
NDGA
CDC
PD
NDGA+CDC
NDGA+PD
CDC+PD
NDGA+CDC+PD
B
Cell proliferation
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Veh
NDGA
CDC
PD
NDGA+CDC
NDGA+PD
CDC+PD
NDGA+CDC+PD
D
Cell proliferation
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Veh
NDGA
CDC
PD
NDGA+CDC
NDGA+PD
CDC+PD
NDGA+CDC+PD

Figure 5.1 Effects of LOX inhibitors on cell viability and proliferation in growing and confluent endothelial cells.

Growing (Panels A and B) and confluent (Panels C and D) EA.hy926 endothelial cells were treated with NDGA (5-LOX inhibitor), CDC (12-LOX inhibitor), and PD (15-LOX inhibitor) individually or in various combinations for 24 h before performing the cell viability (Panels A and C) and cell proliferation assays (Panels B and D). The concentrations of NDGA, CDC and PD used were 2 μM, 1μM and 5μM, respectively. Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from Veh. CDC - cinnamyl-3,4-dihydroxy-α-cyanocinnamate, LOX - lipoxygenase, NDGA - nordihydroguaiaretic acid, PD - PD146176, Veh - vehicle control (DMSO).

Activation of p38 MAPK, Akt and PPARα by PD146176

Since the PD146176 had a significant effect compared to the other two inhibitors, all subsequent experiments were focused on PD146176. To investigate the molecular mechanism of how PD146176 affects endothelial cell viability and proliferation, growing (Figure 5.2) and confluent cells (Figure 5.3) were treated with PD146176 over a concentration range from 1 to 100 μM for both 30 min and 8 h. These time points were selected because p38 MAPK activation had been observed at both early and later times (Chapter 4). In growing cells, Western blotting showed that PD146176 activated p38 MAPK in a dose-dependent manner after 30 min (Figure 5.2A), and that concentrations 20 μM and higher caused significant activation (Figure 5.2B, first panel). In contrast to p38 MAPK activation, PD146176 down-regulated Akt activation in a dose-dependent manner (Figure 5.2B, second panel). A 30 min treatment with PD146176 had no effect on PPARα phosphorylation (Figure 5.2B, third panel). After 8 h (Figure 5.2C), PD146176 up-regulated p38 MAPK (Figure 5.2D, first panel) and down-regulated Akt (Figure 5.2D, second panel) similar to that seen at 30 min. Unlike 30 min, however, at 8 h PPARα phosphorylation was significantly increased with PD146176 concentrations of 5 μM or greater (Figure 5.2D, third panel). Although activation of PPARα with PD146176 at 100 μM was much lower than the other concentrations, it was still significantly different from Veh.

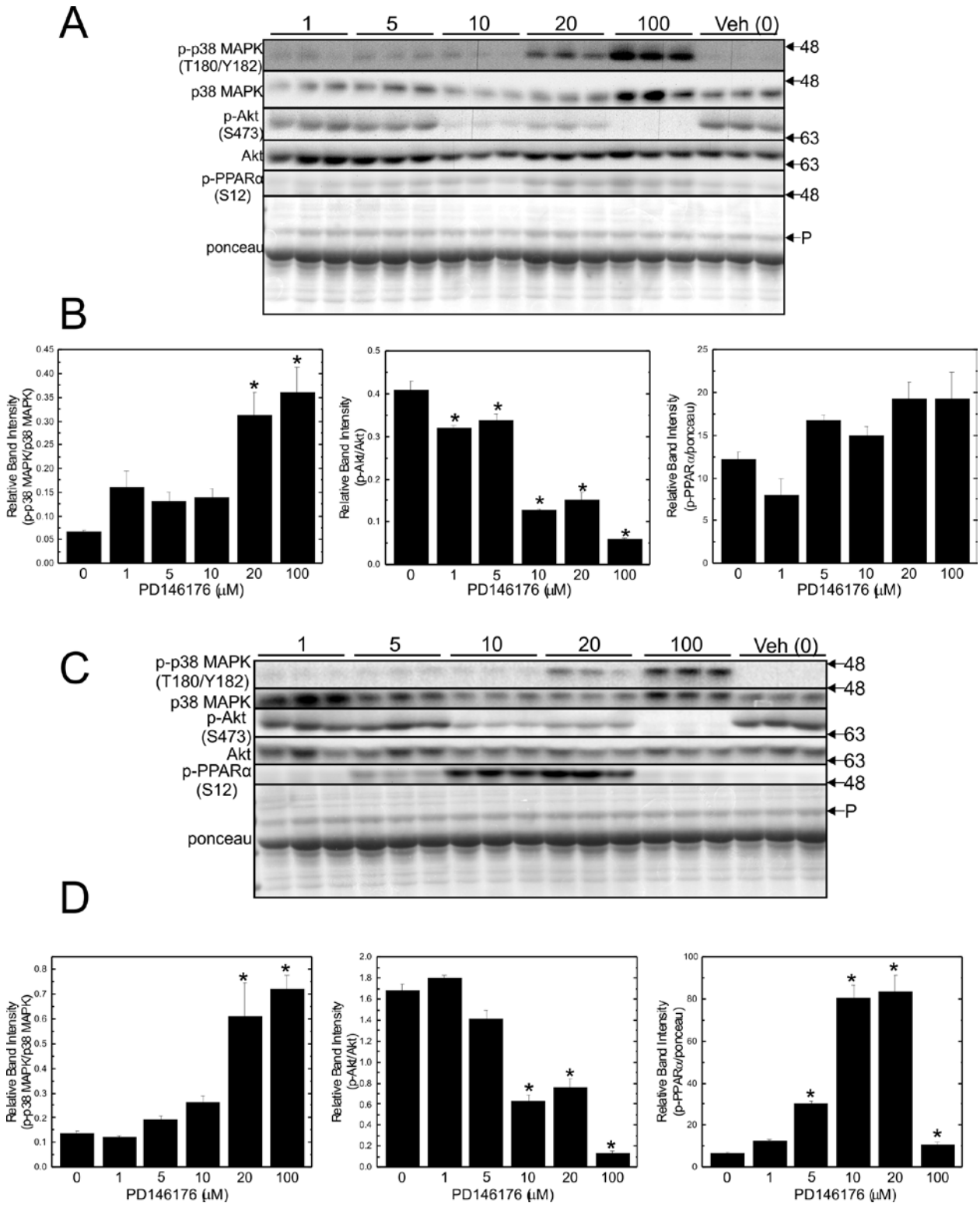

A
1
5
10
20
100
Veh (0)
p-p38 MAPK (T180/Y182)
p38 MAPK
p-Akt (S473)
Akt
p-PPARα (S12)
ponceau
48
48
63
63
48
P
B
Relative Band Intensity (p-p38 MAPK/p38 MAPK)
Relative Band Intensity (p-Akt/Akt)
Relative Band Intensity (p-PPARα/ponceau)
PD146176 (μM)
C
D

Figure 5.2 Effect of PD146176 on p38 MAPK, Akt and PPARα activation in growing cells. Growing EA.hy926 endothelial cells were treated with PD146176 at concentrations from 1 to 100 μM for 30 min (Panels A and B) and 8 h (Panels C and D). Protein levels of p-p38 MAPK, p38 MAPK, p-Akt, Akt, p-PPARα were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panels A and C. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from Veh. Veh - vehicle control (DMSO).

In confluent cells, p38 MAPK phosphorylation was significantly increased with 100 μM PD146176 after 30 min (Figure 5.3A, B first panel) and 8 h (Figure 5.3C, D first panel). In contrast to growing cells, Akt phosphorylation was increased with 5 μM treatment after 30 min (Figure 5.3B, second panel), and with 1 μM and 5 μM treatment after 8 h (Figure 5.3D, second panel). Similar to growing cells, PPARα phosphorylation was not changed after 30 min (Figure 5.3B, third panel), but was increased at 20 μM after 8 h (Figure 5.3D, third panel).

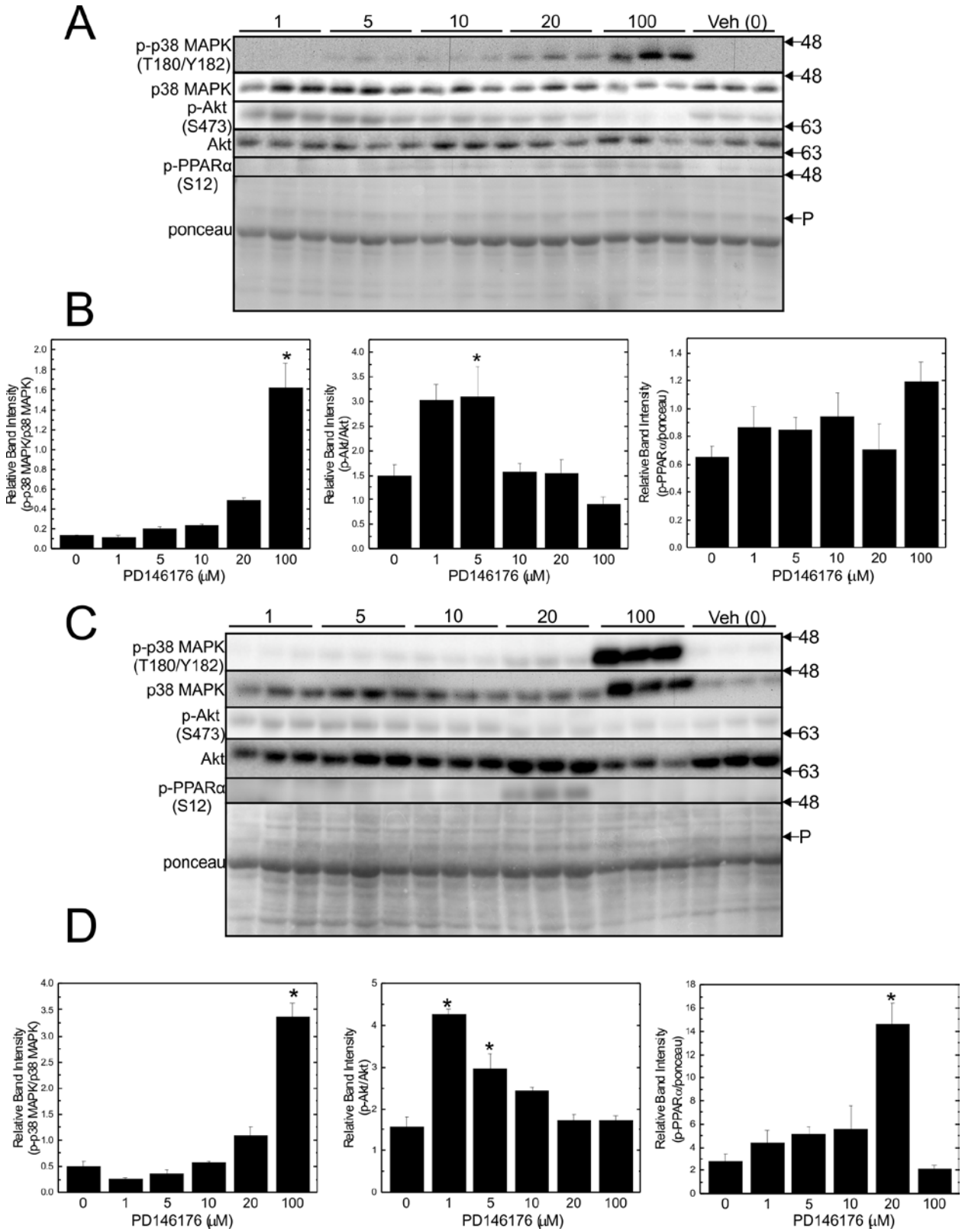

A
1
5
10
20
100
Veh (0)
p-p38 MAPK (T180/Y182)
p38 MAPK
p-Akt (S473)
Akt
p-PPARα (S12)
ponceau
48
48
63
63
48
P
B
Relative Band Intensity (p-p38 MAPK/p38 MAPK)
Relative Band Intensity (p-Akt/Akt)
Relative Band Intensity (p-PPARα/ponceau)
PD146176 (μM)
C
D

Figure 5.3 Effect of PD146176 on p38 MAPK, Akt and PPARα activation in confluent cells. Growing EA.hy926 endothelial cells were treated with PD146176 at concentrations from 1 to 100 μM for 30 min (Panels A and B) and 8 h (Panels C and D). Protein levels of p-p38 MAPK, p38 MAPK, p-Akt, Akt, p-PPARα were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panels A and C. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from Veh. Veh - vehicle control (DMSO).

Cross-talk between p38 MAPK, Akt and PPARα

To study the cross-talk between p38 MAPK, Akt and PPARα after PD146176 treatment, the p38 MAPK inhibitor SB202190 (Figure 5.4A-D) and PPARα antagonist GW6471 (Figure 5.4E-H) were used. Combining SB202190 and PD146176 significantly increased PPARα phosphorylation (Figure 5.4B) compared to PD146176 treatment alone. This indicated that p38 MAPK had an inhibitory effect on PPARα activation by PD146176. Both SB 202190 treatment and the combination treatment of PD146176 and SB202190 significantly decreased Akt activation (Figure 5.4C), but they were not different from PD146176 treatment alone. This indicated there was no cross-talk between p38 MAPK and Akt. Since SB202190 inhibits phosphorylation of p38 MAPK substrates [63], but not phosphorylation of p38 MAPK itself (Figure 5.4B), it was observed that SB 202190 treatment was not different from vehicle for p38 MAPK phosphorylation (Figure 5.4D). However, combining PD146176 and SB202190 significantly increased p38 MAPK phosphorylation compared to PD146176 treatment alone (Figure 5.4D), likely due to the accumulation of phosphorylated p38 MAPK in the combined treatment. Combining GW6471 and PD146176 significantly decreased PPARα activation (Figure 5.4F), and decreased Akt phosphorylation (Figure 5.4G) compared to PD146176 treatment alone. This suggested that GW6471 antagonizes PPARα activity, and PPARα has a stimulatory effect on Akt. p38 MAPK activation was similar with PD146176 treatment alone and the combined PD146176 plus GW6471 treatment, suggesting PPARα was not upstream of p38 MAPK (Figure 5.4H). Figure 5.5 provides an illustration of the signaling of PD146176 with respect to p38 MAPK, PPARα and Akt as determined by these experiments.

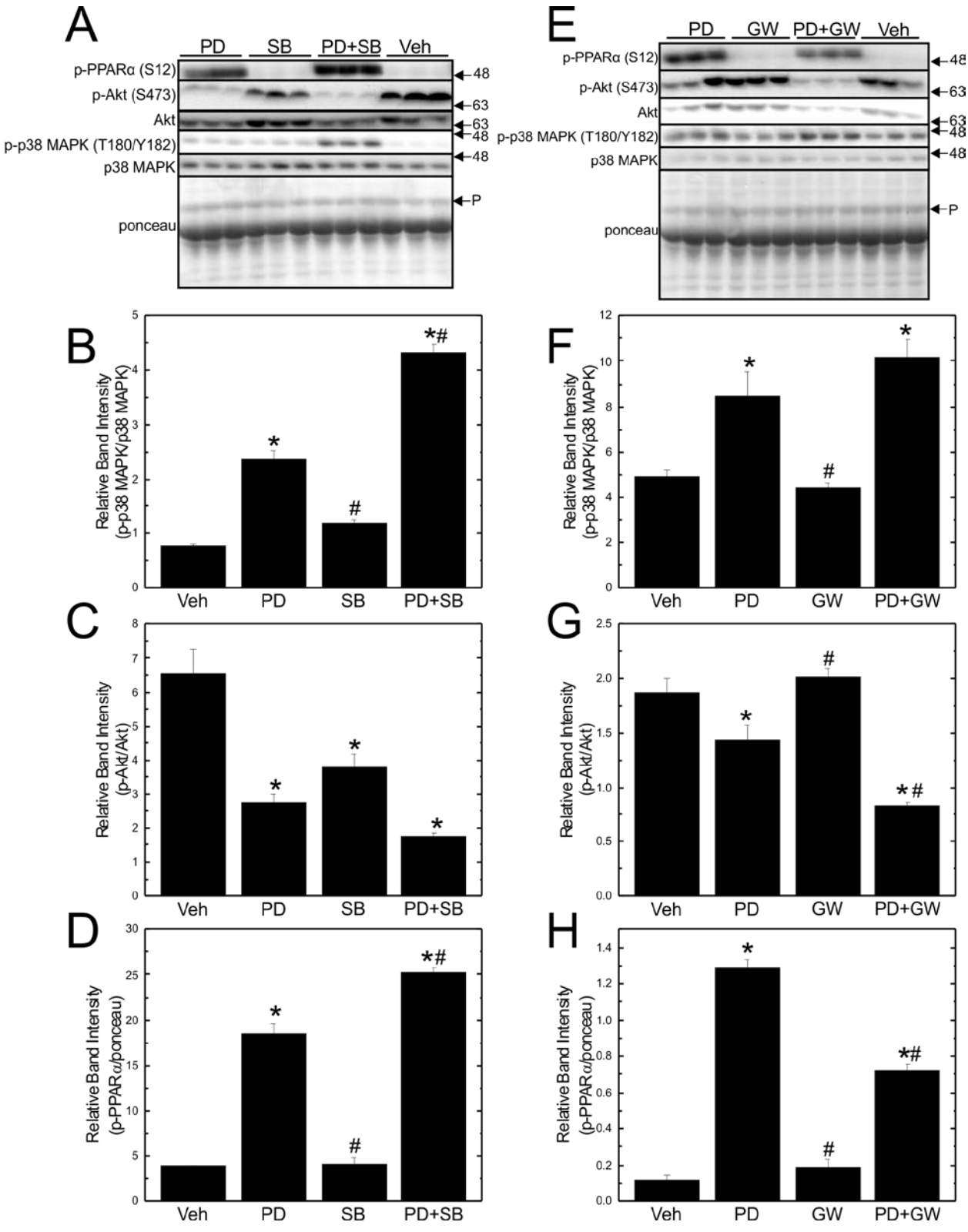

A
PD
SB
PD+SB
Veh
p-PPARα (S12)
p-Akt (S473)
Akt
p-p38 MAPK (T180/Y182)
p38 MAPK
ponceau
48
63
63
48
48
P
E
PD
GW
PD+GW
Veh
p-PPARα (S12)
p-Akt (S473)
Akt
p-p38 MAPK (T180/Y182)
p38 MAPK
ponceau
48
63
63
48
48
P
B
Relative Band Intensity (p-p38 MAPK/p38 MAPK)
Veh
PD
SB
PD+SB
F
Relative Band Intensity (p-p38 MAPK/p38 MAPK)
Veh
PD
GW
PD+GW
C
Relative Band Intensity (p-Akt/Akt)
Veh
PD
SB
PD+SB
G
Relative Band Intensity (p-Akt/Akt)
Veh
PD
GW
PD+GW
D
Relative Band Intensity (p-PPARα/ponceau)
Veh
PD
SB
PD+SB
H
Relative Band Intensity (p-PPARα/ponceau)
Veh
PD
GW
PD+GW

Figure 5.4 Cross-talk between p38 MAPK, Akt and PPARα.

Growing EA.hy926 endothelial cells were treated with PD146176 (25 μM) and SB202190 (1μM) alone or in combination (Panels A-D), and with PD146176 (25 μM) and GW6471 (2.5 μM) alone or in combination (Panels E-H) for 8 h. SB202190 and GW6471 was added to the cells 30 min before adding PD146176 in the combination treatment. Protein levels of p-PPARα, p-Akt, Akt, p-p38 MAPK, p38 MAPK, were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in Panels A and E. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on the image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from Veh. $^{\#}$Treatment significantly different ($p<0.05$) from PD. GW - GW6471; PD - PD146176; SB - SB202190; Veh - vehicle control (DMSO).

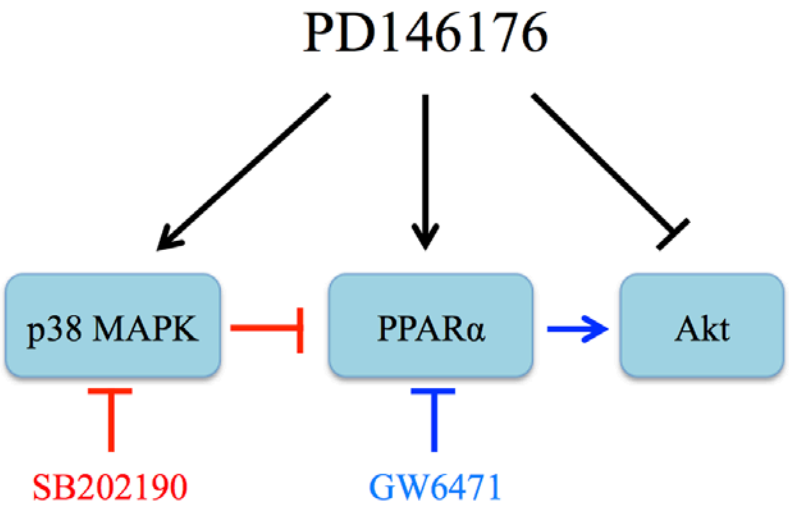


Figure 5.5 Proposed mechanism for the actions of PD146176 (putative 15-LOX inhibitor) on intracellular signaling in EA.hy926 cells.

Oxylipin profile with PD146176 treatment

To examine the changes in oxylipin production that were induced by treatment with PD146176, growing EA.hy926 endothelial cells were treated with 5, 10, 20, 50 and 100 μM PD146176 for 30 min and for 8 h. In total, 71 oxylipins were detected, and are presented in units of picomole per mg protein (Table 5.1). Oxylipins with significant changes were shown in Figure 5.6 (30 min) and Figure 5.7 (8 h). After 30 min of treatment with PD146176, levels of 15-LOX products including 13-HODE, 15-HETE, 12-HETE, 15-HEPE, and 17-HDoHE were not consistent with a dose-dependent inhibition (Table 5.1). On the other hand, while 15-LOX products were unchanged in response to the inhibitor treatment, CYP products from AA including 5,6-EpETrE (Figure 5.6A), 8,9-EpETrE (Figure 5.6B), 11,12-EpETrE (Figure 5.6C) and 14,15-DiHETrE (Figure 5.6D) showed a dose-dependent reduction. 12,13-diHOME (Figure 5.6E), which is a CYP product from LA, was also reduced in a dose-dependent manner with PD146176 treatment. Although a dose-dependent reduction of 16,17-EpDPE (CYP product from DHA) was not obvious, it was significantly reduced by 20 μM PD146176 after 30 min (Figure 5.6F). In contrast, PD146176 at 50 and 100 μM seemed to stimulate the production of 9,10,13-triHOME (Figure 5.6G), an LA-derived oxylipin metabolized from 9-HpODE via 5-LOX. Likewise, at 20 μM, PD146176 increased 13-HDoHE (Figure 5.6H), which is derived non-enzymatically from DHA. Interestingly, 8-HETE (Figure 5.6I), formed non-enzymatically from AA, and 9-HODE (Figure 5.6J), derived from 9-HpODE, were both increased with 20 μM PD146176. These results suggested that PD146176 was not functioning as a 15-LOX inhibitor but was inhibiting oxylipin formation by CYP.

The oxylipins present in the 30 min samples were also detected after 8 h (Table 5.1). Interestingly, the dose-dependent reduction of 5,6-EpETrE, 8,9-EpETrE, 11,12-EpETrE, and 14,15-DiHETrE by PD146176 was not detected at 8 h, except for an elevation in 14,15-

DiHETrE with the 5 and 50 μM treatments (Figure 5.7A). CYP products from LA (9,10-diHOME, Figure 5.7B), AA (11,12-DiHETrE, Figure 5.7C), and DHA (19,20-DiHDoPE, Figure 5.7D) were increased after 8 h at different concentrations of PD146176 depending on the specific oxylipin. A COX product from AA, 15k PGE_2, was also increased by 50 μM PD146176 after 8 h (Figure 5.7F). Interestingly, similar to 30 min treatment, production of 9,10,13-triHOME increased in a dose-dependent manner, reaching a level slightly higher after 8 h (~2.2 pmol/mg protein) (Figure 5.7E) compared to 30 min (~1.8 pmol/mg protein) (Figure 5.6G).

Table 5.1 Oxylipin profile after PD146176 treatment with various concentrations (0-100 µM) in growing cells[1].

Fatty Acid	Pathway	Oxylipin	30 min PD146176 treatment						8 h PD146176 treatment					
			0 uM	5 uM	10 uM	20 uM	50 uM	100 uM	0 uM	5 uM	10 uM	20 uM	50 uM	100 uM
AA	LOX	Tetranor 12-HETE	**0.091** (0.008)	**0.114** (0.047)	**0.101** (0.018)	**0.148** (0.021)	**0.100** (0.018)	**0.060** (0.003)	**0.610** (0.203)	**0.605** (0.146)	**0.498** (0.183)	**0.609** (0.174)	**0.718** (0.066)	**0.307** (0.09)
AA	COX	12-HHTrE	**21.643** (2.589)	**35.056** (21.708)	**29.902** (12.549)	**82.421** (28.921)	**14.614** (5.179)	**4.347** (0.613)	**43.067** (14.595)	**39.388** (11.138)	**23.871** (7.049)	**26.706** (7.49)	**32.335** (1.246)	**12.683** (6.851)
AA	COX	15d PGD_2	**0.305** (0.055)	**0.245** (0.044)	**0.465** (0.164)	**0.524** (0.196)	**0.551** (0.189)	**0.248** (0.042)	**0.337** (0.04)	**0.213** (0.024)	**0.258** (0.052)	**0.450** (0.061)	**0.801** (0.198)	**0.656** (0.247)
AA	COX	15k PGE_2	**0.398** (0.052)	**0.349** (0.043)	**0.458** (0.088)	**0.440** (0.042)	**0.452** (0.115)	**0.443** (0.095)	**0.273** (0.036)	**0.228** (0.034)	**0.253** (0.061)	**0.322** (0.052)	**0.483*** (0.047)	**0.374** (0.062)
AA	COX	dhk PGD_2	**0.119** (0.022)	**0.152** (0.019)	**0.096** (0.003)	**0.103** (0.011)	**0.105** (0.009)	**0.082** (0.023)	**0.095** (0.011)	**0.068** (0.013)	**0.061** (0.006)	**0.090** (0.02)	**0.166** (0.047)	**0.109** (0.011)
AA	COX	PGD_2	**0.652** (0.205)	**0.351** (0.055)	**0.579** (0.1)	**0.357** (0.097)	**0.435** (0.148)	**0.672** (0.24)	**0.253** (0.096)	**0.346** (0.103)	**0.402** (0.215)	**0.391** (0.051)	**0.651** (0.139)	**0.703** (0.298)
AA	COX	PGE_2	**0.200** (0.061)	**0.356** (0.097)	**0.248** (0.039)	**0.296** (0.094)	**0.270** (0.057)	**0.136** (0.065)	**0.319** (0.067)	**0.537** (0.349)	**0.524** (0.334)	**0.271** (0.102)	**0.612** (0.269)	**0.346** (0.112)
AA	COX	$PGF_2\alpha$	**0.334** (0.064)	**0.372** (0.06)	**0.436** (0.082)	**0.414** (0.119)	**0.278** (0.05)	**0.353** (0.149)	**0.399** (0.104)	**0.268** (0.098)	**0.381** (0.184)	**0.300** (0.134)	**0.335** (0.216)	**0.285** (0.068)
AA	COX	TXB_2	**2.012** (0.125)	**1.618** (0.285)	**1.652** (0.141)	**1.529** (0.117)	**1.776** (0.093)	**2.083** (0.142)	**1.616** (0.019)	**2.171** (0.153)	**1.861** (0.228)	**1.875** (0.108)	**2.042** (0.064)	**1.941** (0.067)
AA	CYP	11,12-DiHETrE	**0.135** (0.011)	**0.130** (0.016)	**0.142** (0.01)	**0.122** (0.007)	**0.120** (0.008)	**0.129** (0.015)	**0.069** (0.009)	**0.114** (0.017)	**0.103** (0.018)	**0.111** (0.003)	**0.152*** (0.017)	**0.100** (0.009)
AA	CYP	11,12-EpETrE	**0.395** (0.056)	**0.465** (0.095)	**0.352** (0.014)	**0.234** (0.037)	**0.202** (0.051)	**0.141*** (0.02)	**0.087** (0.021)	**0.168** (0.035)	**0.171** (0.027)	**0.131** (0.014)	**0.189** (0.044)	**0.119** (0.043)
AA	CYP	14,15-DiHETrE	**0.274** (0.013)	**0.240** (0.015)	**0.248** (0.022)	**0.192** (0.024)	**0.145*** (0.027)	**0.115*** (0.014)	**0.121** (0.009)	**0.167*** (0.013)	**0.150** (0.002)	**0.145** (0.008)	**0.186*** (0.016)	**0.122** (0.01)

Table 5.1 Cont'd

Fatty Acid	Pathway	Oxylipin	30 min PD146176 treatment						8 h PD146176 treatment					
			0 uM	5 uM	10 uM	20 uM	50 uM	100 uM	0 uM	5 uM	10 uM	20 uM	50 uM	100 uM
AA	CYP	14,15-EpETrE	**0.357** (0.027)	**0.362** (0.021)	**0.312** (0.019)	**0.262** (0.034)	**0.295** (0.088)	**0.320** (0.052)	**0.066** (0.012)	**0.168** (0.02)	**0.146** (0.039)	**0.162** (0.013)	**0.185** (0.035)	**0.173** **(0.069)**
AA	CYP	16-HETE	**2.138** (0.294)	**2.052** (0.858)	**3.660** (1.309)	**3.012** (0.495)	**2.588** (0.323)	**0.858** (0.139)	**3.069** (0.912)	**2.208** (0.297)	**2.548** (0.261)	**5.304** (1.683)	**5.207** (2.432)	**2.077** **(0.451)**
AA	CYP	17-HETE	**0.211** (0.049)	**0.228** (0.107)	**0.288** (0.118)	**0.298** (0.085)	**0.207** (0.004)	**0.123** (0.008)	**0.295** (0.115)	**0.325** (0.136)	**0.194** (0.053)	**0.243** (0.061)	**0.427** (0.135)	**0.171** **(0.046)**
AA	CYP	18-HETE	**0.108** (0.006)	**0.099** (0.001)	**0.156** (0.05)	**0.135** (0.013)	**0.145** (0.013)	**0.143** (0.011)	**0.162** (0.01)	**0.163** (0.018)	**0.157** (0.005)	**0.169** (0.029)	**0.213** (0.017)	**0.208** **(0.04)**
AA	CYP	19-HETE	**0.255** (0.066)	**0.314** (0.066)	**0.544** (0.185)	**0.515** (0.118)	**0.256** (0.046)	**0.476** (0.139)	**0.223** (0.028)	**0.202** (0.082)	**0.283** (0.113)	**0.198** (0.065)	**0.299** (0.084)	**0.522** **(0.137)**
AA	CYP	20-HETE	**0.043** (0.01)	**0.063** (0.002)	**0.054** (0.005)	**0.042** (0.01)	**0.057** (0.02)	**0.051** (0.01)	**0.026** (0.009)	**0.052** (0.019)	**0.044** (0.018)	**0.046** (0.026)	**0.071** (0.016)	**0.067** **(0.01)**
AA	CYP	5,6-DiHETrE	**1.146** (0.058)	**0.717** (0.103)	**1.070** (0.058)	**0.775** (0.068)	**1.033** (0.096)	**0.980** (0.215)	**0.883** (0.099)	**0.884** (0.113)	**0.847** (0.078)	**0.761** (0.058)	**0.948** (0.216)	**1.091** **(0.132)**
AA	CYP	5,6-EpETrE	**0.260** (0.022)	**0.178*** (0.008)	**0.194*** (0.004)	**0.118*** (0.009)	**0.116*** (0.026)	**0.079*** (0.006)	**0.052** (0.013)	**0.082** (0.013)	**0.081** (0.025)	**0.075** (0.008)	**0.059** (0.007)	**0.051** **(0.013)**
AA	CYP	8,9-DiHETrE	**0.261** (0.015)	**0.249** (0.024)	**0.281** (0.013)	**0.261** (0.02)	**0.224** (0.007)	**0.216** (0.011)	**0.221** (0.016)	**0.237** (0.022)	**0.242** (0.032)	**0.205** (0.017)	**0.279** (0.024)	**0.212** **(0.012)**
AA	CYP	8,9-EpETrE	**0.806** (0.034)	**0.614*** (0.056)	**0.705*** (0.04)	**0.433*** (0.095)	**0.376*** (0.111)	**0.207*** (0.057)	**0.184** (0.036)	**0.355** (0.039)	**0.364** (0.109)	**0.345** (0.031)	**0.283** (0.04)	**0.151** **(0.031)**
AA	LOX	12-HETE	**17.637** (1.059)	**26.082** (11.631)	**20.361** (3.387)	**40.026** (12.707)	**16.494** (0.812)	**14.488** (0.402)	**10.256** (2.559)	**15.100** (3.027)	**12.090** (3.884)	**11.251** (2.065)	**17.770** (1.943)	**12.432** **(1.461)**
AA	LOX	12epi LTB_4	**0.998** (0.091)	**0.989** (0.663)	**1.199** (0.51)	**1.632** (0.778)	**0.371** (0.045)	**0.120** (0.032)	**0.727** (0.284)	**0.932** (0.357)	**0.757** (0.304)	**1.219** (0.206)	**0.630** (0.145)	**0.167** **(0.045)**

Table 5.1 Cont'd

Fatty Acid	Pathway	Oxylipin	30 min PD146176 treatment						8 h PD146176 treatment					
			0 uM	**5 uM**	**10 uM**	**20 uM**	**50 uM**	**100 uM**	**0 uM**	**5 uM**	**10 uM**	**20 uM**	**50 uM**	**100 uM**
AA	LOX	15-HETE	**28.205** (3.365)	**29.343** (8.343)	**53.995** (19.122)	**59.413** (9.154)	**29.319** (2.023)	**15.663** (1.318)	**39.867** (14.449)	**33.541** (7.662)	**26.511** (2.925)	**50.264** (12.642)	**51.631** (10.954)	**19.950** (2.37)
AA	LOX	5-HETE	**31.948** (1.644)	**38.218** (15.875)	**58.095** (22.905)	**122.046** (45.852)	**27.622** (4.288)	**16.442** (2.141)	**52.459** (16.968)	**43.046** (9.129)	**29.411** (6.549)	**54.765** (14.59)	**56.396** (16.05)	**21.140** (3.197)
AA	LOX	5-oxoETE	**20.896** (1.948)	**32.459** (16.029)	**68.097** (27.809)	**35.778** (9.124)	**13.404** (5.532)	**6.393** (2.074)	**12.968** (7.792)	**30.138** (12.486)	**22.101** (14.983)	**32.246** (11.216)	**118.780** (98.98)	**28.246** (15.989)
AA	Non-enz/ LOX	8-HETE	**5.654** (0.098)	**8.925** (3.654)	**9.222** (3.446)	**21.830*** (9.835)	**5.779** (0.439)	**3.297** (0.264)	**6.494** (2.252)	**6.529** (1.559)	**4.987** (1.432)	**7.062** (1.686)	**8.529** (1.68)	**5.106** (0.75)
AA	LOX	LTB_4	**1.089** (0.025)	**1.196** (0.771)	**1.466** (0.673)	**2.453** (1.368)	**0.391** (0.043)	**0.122** (0.028)	**1.028** (0.355)	**1.170** (0.362)	**1.008** (0.432)	**1.381** (0.292)	**0.721** (0.176)	**0.231** (0.045)
AA	non-enz	5-iso $PGF_2\alpha VI$	**0.090** (0.015)	**0.086** (0.019)	**0.122** (0.015)	**0.076** (0.006)	**0.118** (0.015)	**0.097** (0.016)	**0.120** (0.014)	**0.118** (0.023)	**0.082** (0.017)	**0.086** (0.005)	**0.144** (0.027)	**0.115** (0.009)
AA	non-enz /COX/LOX	11-HETE	**3.275** (0.139)	**6.240** (3.762)	**5.879** (2.358)	**15.213** (5.691)	**3.291** (0.336)	**2.215** (0.166)	**5.003** (1.814)	**5.157** (1.385)	**4.069** (1.427)	**5.468** (1.769)	**6.740** (1.384)	**2.905** (0.413)
AA	non-enz /COX/LOX	9-HETE	**5.101** (0.189)	**7.625** (4.305)	**8.878** (4.182)	**32.710** (19.828)	**3.969** (0.157)	**2.237** (0.096)	**6.841** (3.145)	**6.399** (2.881)	**4.594** (1.583)	**6.048** (1.282)	**6.987** (1.407)	**3.211** (0.182)
AA	LOX	12-oxoETE	**33.929** (1.478)	**60.740** (32.237)	**85.882** (36.886)	**92.875** (42.463)	**11.752** (5.469)	**3.063** (0.73)	**29.367** (12.984)	**67.691** (26.277)	**45.073** (28.177)	**38.032** (13.956)	**114.730** (84.405)	**21.476** (12.085)
AA	LOX	15-oxoETE	**20.095** (2.157)	**30.015** (12.733)	**58.817** (23.657)	**48.944** (17.696)	**9.596** (3.814)	**4.847** (1.251)	**12.940** (5.617)	**30.865** (11.696)	**16.339** (10.807)	**27.467** (9.757)	**104.406** (83.028)	**24.134** (13.569)
ALA	LOX	9-HOTrE	**0.262** (0.021)	**0.356** (0.124)	**0.447** (0.12)	**0.663** (0.168)	**0.315** (0.046)	**0.150** (0.01)	**1.324** (0.475)	**0.718** (0.149)	**0.639** (0.078)	**0.941** (0.267)	**0.815** (0.253)	**0.223** (0.03)
DHA	CYP	16,17-EpDPE	**2.168** (0.115)	**2.033** (0.229)	**1.825** (0.228)	**0.865*** (0.227)	**1.520** (0.434)	**1.749** (0.297)	**0.656** (0.108)	**1.419** (0.208)	**1.417** (0.43)	**1.359** (0.1)	**1.001** (0.223)	**0.949** (0.341)

Table 5.1 Cont'd

Fatty Acid	Pathway	Oxylipin	30 min PD146176 treatment						8 h PD146176 treatment					
			0 uM	5 uM	10 uM	20 uM	50 uM	100 uM	0 uM	5 uM	10 uM	20 uM	50 uM	100 uM
DHA	CYP	19,20-DiHDoPE	**0.431** (0.012)	**0.450** (0.037)	**0.433** (0.023)	**0.370** (0.035)	**0.475** (0.028)	**0.519** (0.038)	**0.272** (0.015)	**0.405*** (0.015)	**0.386*** (0.026)	**0.387*** (0.01)	**0.532*** (0.018)	**0.456*** (0.047)
DHA	CYP	19,20-EpDPE	**0.557** (0.032)	**0.579** (0.042)	**0.539** (0.017)	**0.383** (0.036)	**0.536** (0.159)	**0.431** (0.059)	**0.219** (0.03)	**0.460** (0.02)	**0.405** (0.085)	**0.483** (0.066)	**0.302** (0.087)	**0.310** (0.115)
DHA	LOX	10-HDoHE	**3.448** (0.048)	**3.703** (1.403)	**4.130** (1.044)	**5.835** (0.973)	**2.760** (0.209)	**2.017** (0.134)	**5.680** (2.221)	**4.923** (0.6)	**7.165** (2.762)	**6.691** (2.107)	**5.902** (1.208)	**2.599** (0.315)
DHA	LOX	11-HDoHE	**2.748** (0.1)	**5.724** (3.783)	**4.529** (1.661)	**9.854** (3.486)	**2.250** (0.157)	**1.699** (0.085)	**6.829** (3.01)	**6.982** (2.26)	**5.366** (1.99)	**5.365** (1.782)	**4.183** (1.073)	**1.904** (0.103)
DHA	LOX	13-HDoHE	**5.340** (0.146)	**7.906** (3.491)	**7.723** (2.24)	**21.062*** (6.65)	**4.977** (0.16)	**3.660** (0.312)	**10.022** (4.202)	**11.714** (3.555)	**10.986** (4.036)	**11.904** (4.045)	**9.527** (1.952)	**4.161** (0.225)
DHA	LOX	14-HDoHE	**6.009** (0.638)	**6.095** (1.537)	**9.216** (2.994)	**9.222** (1.684)	**5.693** (0.307)	**3.695** (0.272)	**7.137** (2.26)	**7.390** (1.462)	**6.008** (1.267)	**7.060** (1.892)	**5.797** (1.6)	**3.599** (0.387)
DHA	LOX	17-HDoHE	**11.379** (0.65)	**11.960** (2.578)	**18.608** (7.596)	**17.585** (2.964)	**9.736** (0.146)	**7.550** (0.618)	**16.438** (4.793)	**20.626** (6.126)	**13.032** (2.634)	**15.405** (3.049)	**13.084** (2.001)	**6.581** (0.295)
DHA	LOX	17k DHA	**107.667** (5.814)	**63.764** (26.863)	**366.195** (152.885)	**447.014** (231.898)	**65.784** (18.287)	**38.029** (6.827)	**236.228** (148.265)	**354.136** (158.049)	**198.625** (153.799)	**373.510** (155.672)	**1041.734** (756.377)	**215.160** (145.563)
DHA	LOX	4-HDoHE	**34.987** (0.242)	**35.800** (10.088)	**51.050** (13.749)	**61.705** (15.9)	**27.838** (3.283)	**18.159** (1.837)	**70.355** (22.408)	**49.143** (4.245)	**38.967** (3.893)	**64.764** (14.486)	**40.441** (12.737)	**19.467** (2.221)
DHA	LOX	7-HDoHE	**5.405** (0.169)	**7.117** (3.621)	**6.734** (2.287)	**12.378** (3.704)	**3.625** (0.354)	**2.424** (0.149)	**9.074** (3.282)	**7.316** (1.451)	**8.375** (3.262)	**9.371** (2.381)	**6.805** (1.258)	**3.212** (0.408)
DHA	non-enz/CYP	20-HDoHE	**6.161** (1.266)	**4.760** (0.474)	**10.091** (3.416)	**7.398** (1.809)	**6.224** (0.251)	**7.336** (1.848)	**8.011** (0.944)	**9.174** (2.093)	**6.479** (1.667)	**8.179** (1.317)	**5.311** (1.445)	**5.624** (0.829)
DHA	non-enz/LOX	16-HDoHE	**3.883** (0.116)	**4.388** (1.114)	**6.307** (2.268)	**6.424** (1.127)	**3.486** (0.061)	**2.671** (0.149)	**6.576** (2.04)	**6.532** (1.153)	**5.426** (1.05)	**7.112** (1.48)	**5.200** (0.977)	**2.715** (0.255)

Table 5.1 Cont'd

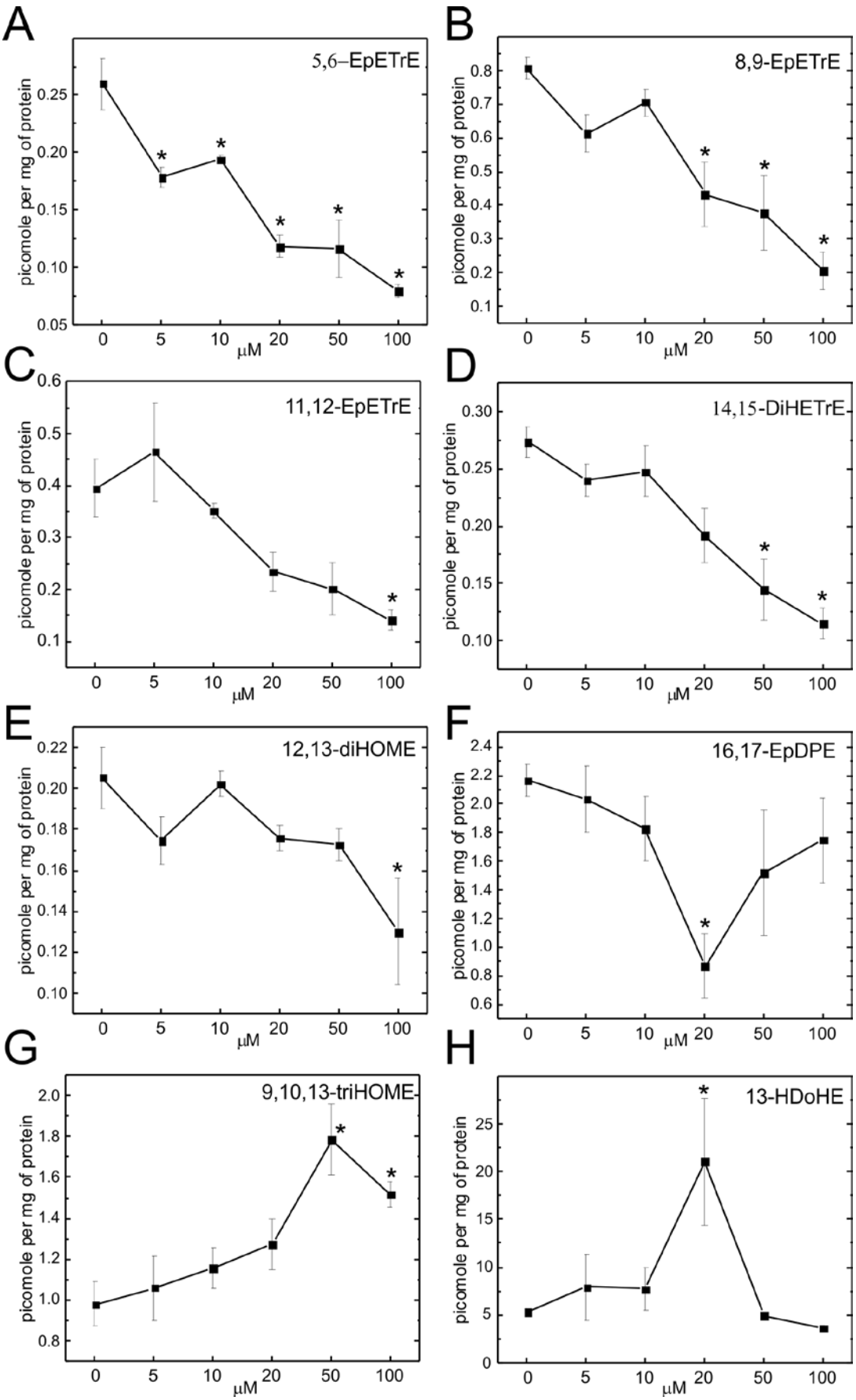
Fatty Acid	Pathway	Oxylipin	30 min PD146176 treatment						8 h PD146176 treatment					
			0 uM	**5 uM**	**10 uM**	**20 uM**	**50 uM**	**100 uM**	**0 uM**	**5 uM**	**10 uM**	**20 uM**	**50 uM**	**100 uM**
DHA	non-enz/LOX	8-HDoHE	**6.024** (0.247)	**6.876** (1.879)	**8.357** (2.123)	**16.736** (7.547)	**5.348** (0.158)	**3.684** (0.204)	**16.949** (8.278)	**9.861** (1.687)	**7.478** (1.08)	**12.719** (2.311)	**10.538** (3.162)	**5.447** (0.517)
DγLA	COX	15k PGE_1	**0.352** (0.027)	**0.356** (0.041)	**0.356** (0.063)	**0.385** (0.057)	**0.302** (0.033)	**0.327** (0.035)	**0.289** (0.026)	**0.266** (0.044)	**0.283** (0.064)	**0.298** (0.044)	**0.329** (0.038)	**0.256** (0.012)
DγLA	LOX	15-HETrE	**4.476** (0.82)	**4.367** (1.552)	**7.919** (2.873)	**8.293** (1.718)	**4.162** (0.251)	**1.916** (0.283)	**6.431** (2.302)	**5.656** (1.538)	**4.513** (0.745)	**7.689** (2.299)	**6.246** (2.009)	**2.455** (0.264)
DγLA	LOX	8-HETrE	**3.061** (0.167)	**3.776** (1.533)	**6.155** (2.539)	**13.605** (5.377)	**2.791** (0.375)	**1.365** (0.149)	**5.483** (1.916)	**4.938** (1.147)	**2.998** (0.579)	**6.161** (1.69)	**4.959** (1.494)	**1.602** (0.209)
EPA	COX	TXB_3	**13.318** (1.336)	**10.619** (1.278)	**16.347** (2.398)	**17.321** (2.295)	**18.981** (6.999)	**13.322** (3.015)	**14.172** (1.531)	**10.145** (0.594)	**14.894** (4.118)	**20.893** (0.856)	**31.984** (10.45)	**16.483** (5.936)
EPA	CYP	18-HEPE	**2.223** (0.183)	**2.274** (0.81)	**3.642** (1.363)	**3.460** (0.262)	**2.156** (0.307)	**1.273** (0.265)	**4.437** (1.355)	**3.503** (0.571)	**2.657** (0.271)	**4.399** (1.073)	**3.843** (0.76)	**1.916** (0.217)
EPA	LOX	12-HEPE	**1.535** (0.094)	**2.213** (0.873)	**1.956** (0.509)	**4.844** (1.884)	**1.531** (0.136)	**1.231** (0.018)	**1.943** (0.644)	**2.009** (0.499)	**1.407** (0.46)	**1.433** (0.419)	**1.978** (0.241)	**1.322** (0.104)
EPA	LOX	15-HEPE	**0.625** (0.107)	**0.774** (0.338)	**1.203** (0.564)	**1.145** (0.429)	**0.606** (0.01)	**0.396** (0.017)	**0.650** (0.204)	**0.530** (0.096)	**0.644** (0.232)	**0.683** (0.208)	**0.827** (0.249)	**0.543** (0.021)
EPA	LOX	5-HEPE	**1.909** (0.389)	**1.624** (0.325)	**3.009** (0.829)	**4.841** (1.642)	**1.887** (0.65)	**1.266** (0.249)	**3.191** (0.352)	**2.768** (0.268)	**2.178** (0.522)	**4.081** (0.679)	**2.910** (0.802)	**1.790** (0.607)
EPA	LOX	8-HEPE	**0.664** (0.097)	**0.819** (0.266)	**1.083** (0.38)	**1.530** (0.582)	**0.614** (0.141)	**0.423** (0.043)	**1.850** (1.091)	**0.806** (0.069)	**0.449** (0.012)	**1.346** (0.355)	**1.665** (0.577)	**0.637** (0.086)
EPA	non-enz/COX/LOX	9-HEPE	**0.747** (0.12)	**0.758** (0.29)	**0.820** (0.329)	**1.543** (0.634)	**0.620** (0.036)	**0.332** (0.031)	**1.219** (0.532)	**0.828** (0.125)	**0.889** (0.402)	**1.063** (0.301)	**1.129** (0.541)	**0.758** (0.117)
LA	CYP	12,13-diHOME	**0.205** (0.015)	**0.175** (0.012)	**0.202** (0.006)	**0.176** (0.006)	**0.172** (0.008)	**0.130*** (0.026)	**0.176** (0.009)	**0.233** (0.007)	**0.206** (0.012)	**0.174** (0.005)	**0.217** (0.005)	**0.163** (0.03)

Table 5.1 Cont'd

Fatty Acid	Pathway	Oxylipin	30 min PD146176 treatment						8 h PD146176 treatment					
			0 uM	5 uM	10 uM	20 uM	50 uM	100 uM	0 uM	5 uM	10 uM	20 uM	50 uM	100 uM
LA	CYP	12,13-EpOME	**0.129** (0.01)	**0.117** (0.006)	**0.111** (0.002)	**0.089** (0.013)	**0.094** (0.026)	**0.121** (0.016)	**0.066** (0.011)	**0.129** (0.01)	**0.105** (0.023)	**0.095** (0.009)	**0.081** (0.014)	**0.086** (0.029)
LA	CYP	9,10-diHOME	**0.169** (0.015)	**0.165** (0.011)	**0.184** (0.011)	**0.161** (0.018)	**0.200** (0.006)	**0.170** (0.013)	**0.152** (0.004)	**0.188** (0.001)	**0.188** (0.01)	**0.204*** (0.003)	**0.233*** (0.019)	**0.183** (0.014)
LA	CYP	9,10-EpOME	**0.070** (0.008)	**0.061** (0.004)	**0.065** (0.004)	**0.065** (0.009)	**0.065** (0.014)	**0.064** (0.011)	**0.035** (0.008)	**0.072** (0.011)	**0.073** (0.022)	**0.075** (0.005)	**0.068** (0.015)	**0.063** (0.018)
LA	LOX	13-HODE	**7.287** (0.106)	**6.648** (1.424)	**8.594** (1.703)	**11.610** (1.435)	**7.494** (1.224)	**4.561** (0.53)	**17.153** (3.013)	**14.979** (2.623)	**11.222** (0.524)	**19.643** (6.712)	**16.815** (5.768)	**6.541** (1.374)
LA	LOX	13-oxoODE	**10.409** (1.305)	**19.321** (9.181)	**45.941** (24.723)	**127.290** (78.426)	**4.995** (1.427)	**2.457** (0.643)	**35.421** (23.665)	**69.750** (41.39)	**30.911** (21.907)	**39.365** (13.307)	**104.505** (68.301)	**36.377** (29.858)
LA	LOX/ COX/CYP	9-HODE	**9.999** (0.698)	**11.424** (4.188)	**19.287** (7.319)	**31.263*** (7.679)	**10.729** (1.895)	**5.982** (0.836)	**54.464** (22.265)	**31.472** (7.734)	**23.531** (3.847)	**39.388** (10.441)	**37.559** (7.772)	**9.640** (1.997)
LA	LOX	9-oxoODE	**9.082** (1.085)	**13.939** (6.691)	**34.643** (14.603)	**26.040** (9.238)	**6.569** (2.319)	**3.098** (0.8)	**14.920** (7.672)	**38.308** (15.123)	**20.400** (12.876)	**32.064** (10.923)	**97.433** (77.94)	**22.820** (13.608)
LA	LOX /CYP	9,10,13-triHOME	**0.979** (0.108)	**1.058** (0.16)	**1.156** (0.103)	**1.272** (0.125)	**1.786*** (0.172)	**1.516*** (0.063)	**1.074** (0.158)	**1.361** (0.071)	**1.430** (0.154)	**1.851*** (0.258)	**2.181*** (0.229)	**2.145*** (0.103)
LA	LOX /CYP	9,12,13-triHOME	**0.483** (0.058)	**0.532** (0.075)	**0.478** (0.027)	**0.476** (0.07)	**0.553** (0.069)	**0.661** (0.18)	**0.484** (0.062)	**0.583** (0.013)	**0.528** (0.117)	**0.562** (0.098)	**0.671** (0.112)	**0.638** (0.064)
MA	LOX	5-HETrE	**2.737** (0.294)	**2.963** (1.05)	**5.148** (1.855)	**11.770** (5.546)	**2.293** (0.302)	**1.370** (0.276)	**4.171** (1.194)	**3.480** (0.726)	**2.465** (0.495)	**4.832** (0.965)	**3.594** (1.209)	**1.595** (0.318)
GLA	LOX	13-HOTrE-γ	**0.264** (0.034)	**0.287** (0.087)	**0.242** (0.031)	**0.363** (0.057)	**0.302** (0.012)	**0.207** (0.014)	**0.698** (0.266)	**0.591** (0.187)	**0.516** (0.035)	**0.921** (0.298)	**0.893** (0.216)	**0.366** (0.024)

[1] Growing EA.hy926 endothelial cells were treated with PD146176 at concentrations of 5, 10, 20, 50 and 100 μM for 30 min or for 8 h. Media was collected and subjected to SPE and HPLC-MS/MS for oxylipin analysis. Data are reported as mean (SEM), n=3 wells; units are pmole/mg of protein. *$p<0.05$, versus 0 μM (Veh) within a time point; gray shading indicates data with statistical significance.

Abbreviations: AA - arachidonic acid; COX - cyclooxygenase, CYP - cytochrome P450, DγLA, dihomo-γ-linoleic acid, EPA, eicosapentaenoic acid, DHA, docosahexaenoic acid, DiHDoPE - dihydroxy- docosapentaenoic acid, DiHETrE - dihydroxy-eicosatrienoic acid, diHOME - dihydroxy-octadecenoic acid, EpDPE - epoxy- docosapentaenoic acid, EpETrE - epoxy-eicosatrienoic acid, EpOME - epoxy- octadecenoic acid, GLA - γ linoleic acid, HDoHE - hydroxyl - docosahexaenoic acid, HEPE - hydroxy- eicosapentaenoic acid, HETE - hydroxy-eicosatetraenoic acid; HETrE - hydroxy-eicosatrienoic acid, HHTrE - hydroxy-heptadecatrienoic acid, HODE - hydroxy-octadecadienoic acid, HOTrE - hydroxy-octadecatrienoic acid, LA, linoleic acid, LOX - lipoxygenase, LT - leukotriene, MA - mead acid, non-enz - non-enzymatic, oxoETE - oxo- eicosatetraenoic acid, oxoODE - oxo-octadecadienoic acid, PG - prostaglandin, SPE, solid phase extraction, triHOME - trihydroxy-octadecenoic acid, TX - thromboxane.


A
5,6–EpETrE
picomole per mg of protein
0.25
0.20
0.15
0.10
0.05
0
5
10
20
50
100
μM
B
8,9-EpETrE
picomole per mg of protein
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
20
50
100
μM
C
11,12-EpETrE
picomole per mg of protein
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
20
50
100
μM
D
14,15-DiHETrE
picomole per mg of protein
0.30
0.25
0.20
0.15
0.10
0
5
10
20
50
100
μM
E
12,13-diHOME
picomole per mg of protein
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0
5
10
20
50
100
μM
F
16,17-EpDPE
picomole per mg of protein
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0
5
10
20
50
100
μM
G
9,10,13-triHOME
picomole per mg of protein
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
5
10
20
50
100
μM
H
13-HDoHE
picomole per mg of protein
25
20
15
10
5
0
0
5
10
20
50
100
μM

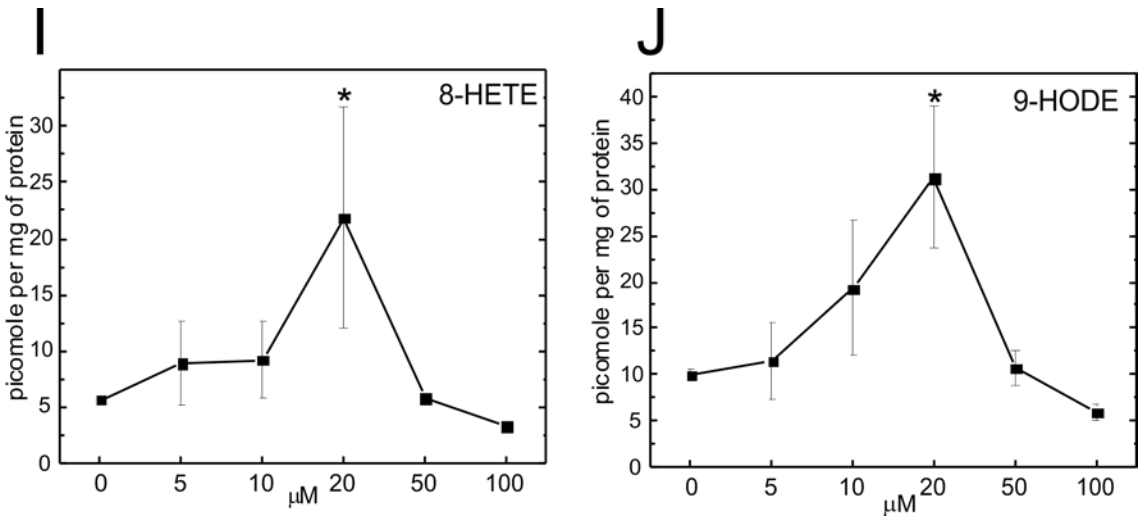


Figure 5.6 Oxylipins with significant changes after 30 min of PD146176 treatment.

Growing EA.hy926 endothelial cells were treated with PD146176 at concentrations of 5, 10, 20, 50 and 100 μM for 30 min. Media was collected at 30 min and subjected to SPE and HPLC-MS/MS for oxylipin analysis. Data are plotted as means ± SEM (n=3 wells). *Significantly different ($p<0.05$) from Veh (0 μM). See Table 5.1 for abbreviations.

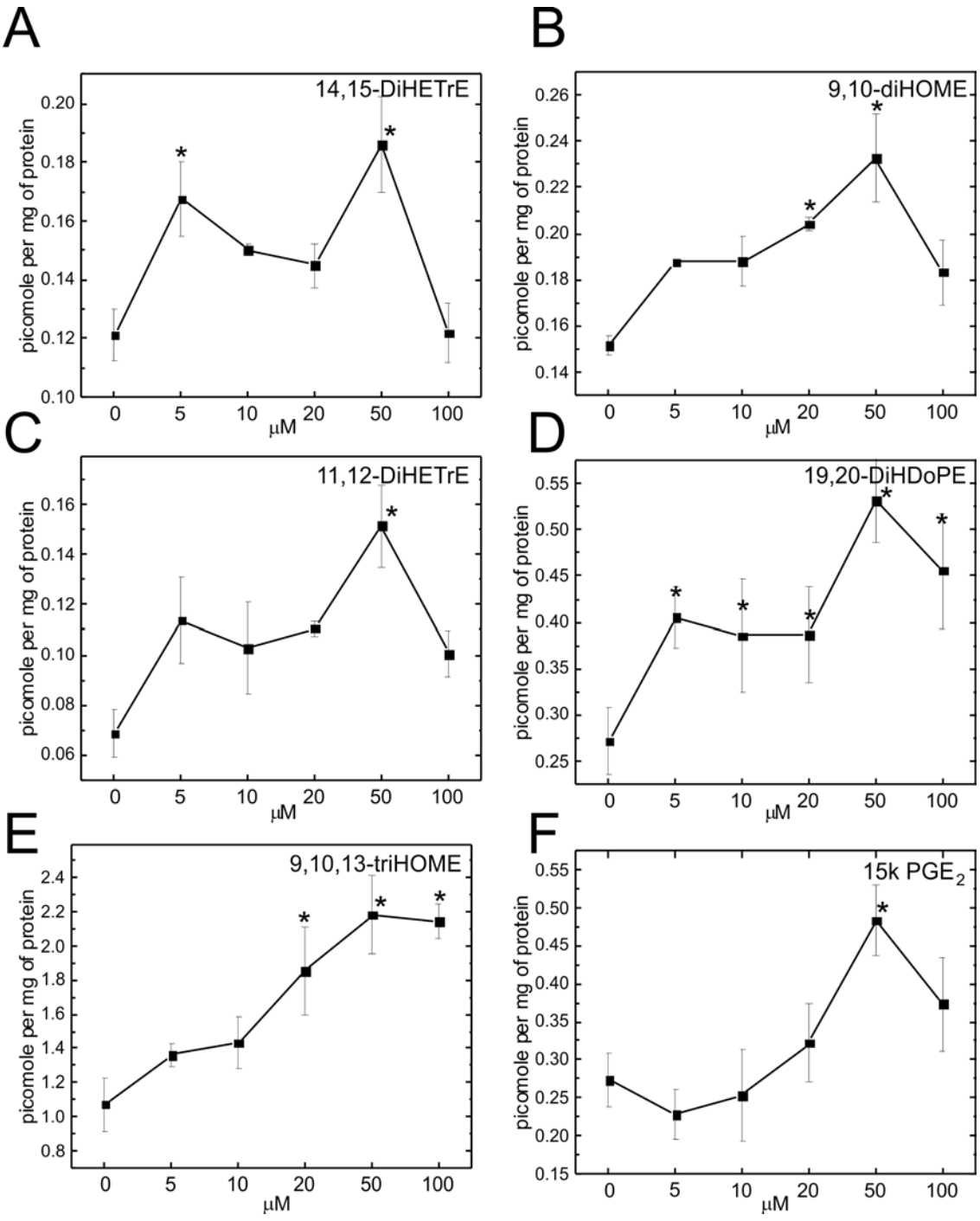

A
14,15-DiHETrE
picomole per mg of protein
0.10
0.12
0.14
0.16
0.18
0.20
0
5
10
20
50
100
μM
B
9,10-diHOME
picomole per mg of protein
0.14
0.16
0.18
0.20
0.22
0.24
0.26
0
5
10
20
50
100
μM
C
11,12-DiHETrE
picomole per mg of protein
0.06
0.08
0.10
0.12
0.14
0.16
0
5
10
20
50
100
μM
D
19,20-DiHDoPE
picomole per mg of protein
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0
5
10
20
50
100
μM
E
9,10,13-triHOME
picomole per mg of protein
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
0
5
10
20
50
100
μM
F
15k PGE2
picomole per mg of protein
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
0
5
10
20
50
100
μM

Figure 5.7 Oxylipins with significant changes after 8 h of PD146176 treatment.

Growing EA.hy926 endothelial cells were treated with PD146176 at concentrations of 5, 10, 20, 50 and 100 μM for 8 h. Media was collected and subjected to SPE and HPLC-MS/MS for oxylipin analysis. Data are plotted as means ± SEM (n=3 wells). *Significantly different ($p<0.05$) from Veh (0 μM). See Table 5.1 for abbreviations.

5.7 Discussion

Our investigation has shown for the first time that compound PD146176 reduced the viability of both growing and confluent EA.hy926 endothelial cells, and decreased the proliferation rate of growing cells. At the molecular level, we found that PD146176 activated p38 MAPK and PPARα, while down-regulating Akt. Furthermore, p38 MAPK had an inhibitory effect on PPARα, while PPARα had a stimulatory effect on Akt, which suggests that a linear pathway links these three signaling proteins. PD146176, which was selected because of reports that it is an effective 15-LOX inhibitor, did not block the formation of 15-LOX products in EA.hy926 endothelial cells. Rather, PD146176 inhibited production of several CYP products from AA such as 5,6-, 8,9-, 11,12-EpETrE and 14,15-DiHETrE, and from LA such as 12,13-diHOME 30 min after treatment, and increased production of 8-HETE and 9-HODE. While these effects were not seen after 8 h, PD146176 induced a dose-dependent increase of other CYP products including 9,10-diHOME, 11,12-DiHETrE, 14,15-DiHETrE, 19,20-DiHDoPE and 9,10,13-triHOME, and one COX product, 15k PGE_2. These data indicate oxylipins originating via CYP are likely key factors in the regulation of endothelial cell processes associated with proliferation and viability. Alternatively, the effect of the inhibitor on cell viability and proliferation may explain the changes in CYP oxylipin levels.

Oxylipins derived from 5-, 12-, and 15-LOX were detected at basal conditions (Table 5.1), which indirectly indicated the presence and activity of the three LOX isoforms in EA.hy926 cells. Although the negative effects of PD146176 were apparently not due to its inhibition of 15-LOX, 15-LOX is well established in the pathogenesis of vascular disease as it relates to diabetic retinopathy [51], atherosclerotic lesion development in animals [64, 65] and humans [66], LDL oxidation [67], and VCAM-1 expression in response to TNF-α treatment [68]. Overexpression of

15-LOX in the LDL-receptor knockout mouse produced larger atherosclerotic lesions at the aortic sinus than in mice with just LDL-receptor deficiency [69].

PD146176 was shown to inhibit 15-LOX activity in rabbit reticulocytes with an IC_{50} of about 0.54 μM, and an IC_{50} of 0.81 μM in intact IC21 cells transfected with 15-LOX [62]. Based on these data, it has been assumed that the effect of PD146176 on various cell processes, including development of dietary cholesterol-induced atherosclerotic lesions in rabbits [70] and fibrous and fibrofoamy plaque development [62], was due to 15-LOX. However, studies of the direct effect of PD146176 on endothelial cell function are very limited. We have shown that PD146176 at a concentration of 5 μM (10× IC_{50} of 0.54 μM), reduced the cell viability of growing and confluent endothelial cells, and reduced the proliferation rate of growing cells. Sendobry et al. [70] also showed that endothelial cells exhibited intense immunostaining of 15-LOX in atherosclerotic lesions, while PD146176-treated animals showed much less 15-LOX immunostaining. In another study, PD146176 was shown to prevent TNF-α/cyclohexamide-induced apoptosis in BAECs with selenium deficiency (an oxidative stress model), while addition of 15-HpETE, which is produced from AA, partially restored apoptosis [71]. However, whether PD146176 induced a reduction of 15-HpETE was not tested. Based on the examples given above, it has been assumed that the beneficial effect of PD146176 is due to inhibition of 15-LOX [62, 70, 71]. However, the major limitation of these studies was the failure to monitor 15-LOX product formation, and thus it remains unclear whether the effects of PD146176 are mediated via 15-LOX. This discrepancy is likely due to the non-specific effects typically encountered when using pharmacological inhibitors.

This is the first report that PD146176 activates p38 MAPK and PPARα in both growing and confluent endothelial cells, while concurrently down-regulating Akt phosphorylation. We showed that p38 MAPK could inhibit PPARα activity, while PPARα had a stimulatory effect on

Akt phosphorylation. In contrast, other studies have suggested the opposite, since p38 MAPK directly phosphorylates PPARα in rat cardiac myocyte [72]. As well, inhibition of p38 MAPK does not block PPARα gene expression in primary rat astrocytes, and p38 MAPK stabilizes the half-life of PPARα mRNA [73]. PPARα inhibited vascular endothelial growth factor-induced endothelial cell migration through inhibiting Akt phosphorylation [74], and overexpression of PPARα inhibited endothelin-induced Akt activation [75]. In our study, we found an activation of p38 MAPK coincides with a reduced production of anti-inflammatory EpETrEs such as 5,6-, 8,9- and 11,12-EpETrEs. This may indicate that loss of the anti-inflammatory EpTrEs caused the activation of p38 MAPK, which in turn negatively affected cell viability. EpETrEs also have pro-angiogenic effect [76]; lower levels of EpETrEs after PD146176 treatment may cause the reduction in cell proliferation that was observed in this study. 13-HDoHE, which can be produced by COX [77], is also an auto-oxidative product generated from DHA that is an indicator of oxidative stress [78]. An elevated level of 13-HDoHE after 30 min treatment with PD146176 may indicate higher levels of oxidative stress in these cells, which may help explain why p38 MAPK activation occurred at the same time since reactive oxygen species can activate p38 MAPK [79].

Decreased Akt phosphorylation was observed after PD146176 treatment of growing cells, in parallel with the reduction in cell viability and proliferation. In contrast to growing cells, Akt phosphorylation was slightly increased after both 30 min and 8 h in confluent cells. Although the viability of confluent cells was reduced, the proliferation rate was unaffected. These contrasting results likely indicate that the mechanisms by which Akt affects cell viability are different in growing versus confluent cells.

With respect to the weak and strong activation of PPARα that was observed 30 min and 8 h, respectively, after PD146176 treatment, it is plausible that the elevated production of 8-HETE,

11,12-, and 14,15-DiHETrE is responsible for modulating PPARα. This interpretation is based on previous reports that these oxylipins are known ligands of PPARα [57, 80]. However, based on these data it is not possible to determine whether p38 MAPK and Akt activation are responsible for the change in DiHETrE levels or whether a second activation step involving phosphorylation by Akt is necessary for activating PPARα.

A reduction in 5,6-, 8,9-, 11,12-EpETrE, 14,15-DiHETrE, 12,13-diHOME, and 16,17-EpDPE was observed in our study 30 min after PD146176 treatment. EpETrEs have pro-angiogenic effect [76]; specifically, 8,9-, and 11,12-EpETrE were reported to increase the BAEC proliferation in a dose-dependent manner [81], while 11,12-EpETrE was shown to increase HUVEC proliferation [82]. The reduction of these oxylipins by PD146176 may explain the lower proliferation rate that was observed. 16,17-EpDPE reduced angiogenesis *in vivo* [82]. It is concluded in the literature that EpETrE products from AA are angiogenic while EpDPE from DHA are anti-angiogenic. Based on these findings, the observed reduction of EpETrE and EpDPE may be the cause of the overall decline in endothelial cell proliferation as presented in this study. 9,10,13-triHOME was also elevated with PD146176 treatment, however, its actions on endothelial cells is unknown.

It is noted that after 8 h of PD146176 treatment, the inhibitory effect on oxylipin production was lost. Instead, at that time point, some products started accumulating. These include 11,12- and 14,15-DiHETrE (which were produced from 11,12-and 14,15-EpETrE via soluble epoxide hydrolase [sEH], respectively), which may suggest an increase in the activity of sEH. Studies on the effect of 9,10-diHOME, 11,12-DiHETrE, 14,15-DiHETrE and 19,20-DiHDoPE on endothelial cell viability and proliferation are rare. However, an EpETrE product from AA is believed to exert an anti-inflammatory effect on monocytes and macrophages [83], and high endogenous levels of CYP products help reduce diabetes [84], atherosclerosis [85], and

dyslipidemia [85] in various mouse models. Although their effects on endothelial cells are not fully understood, several studies have shown that inhibition of epoxygenase in EA.hy926 endothelial cells induced TNF-α release [86], and EpETrEs are considered anti-inflammatory due to their ability to inhibit NF-κB activation in endothelial cells [87, 88]. Lee et al. [89] showed in a transgenic mouse model that endothelial production of human EpETrEs from CYP2J2 and CYP2C8 exerted hypotensive effects. Hu et al. [90] also showed that 19,20-DiHDopE disrupted HUVEC VE-cadherin continuity and induced an increase in permeability indicating the junctions have been perturbed. With this evidence from the literature, our results may suggest that after 8 h treatment with PD146176, endothelial cells became pro-inflammatory, producing high levels of dihydroxy-products such as DiHETrE and DiHDoPE, thus inducing p38 MAPK activation.

5.8 Conclusion

To conclude, our study is the first to report that PD146176, which has been commonly used as a 15-LOX specific inhibitor, did not prevent the synthesis of known 15-LOX oxylipins in EA.hy926 endothelial cells compared to their baseline oxylipin profile at concentration of 5 to 100 μM. However, PD146176 reduced the production of several CYP products at 30 min and induced the accumulation of another group of CYP products at 8 h. These oxylipins may mediate the negative effects of PD146176 on endothelial cell viability and proliferation. This modulation of endothelial cells might occur via regulation of p38 MAPK, PPARα and Akt signaling. Future studies to find specific endothelial LOX inhibitors are warranted and it is suggested that measuring the levels of 15-LOX products should be done when using compounds such as PD146176 to ensure attribution of their effects on cells.

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Chapter 6: Discussion

6.1 Discussion

The focus of this thesis was to investigate the effect of various dietary PUFAs (LA, AA, ALA, EPA, DHA) on EA.hy926 human endothelial cell viability and proliferation, and it was determined that changes in these parameters were not only PUFA-specific, but also dependent on growth state and extracellular substrate. In large part, it was identified that these effects of PUFAs were mediated by the growth state-dependent activation of MAPK (p38, ERK1/2, JNK). Activation of p38, ERK1/2 and JNK by DHA in both growing and confluent endothelial cells followed different temporal sequences. Additionally, it was concluded that PD146176 does not inhibit 15-LOX, but instead affects CYP in EA.hy926 cells. Finally, growth state was found to be involved in the effect of PD146176 on cell viability and proliferation, and this effect was possibly mediated by CYP-derived oxylipins and by modulation of p38 MAPK, Akt and PPARα-dependent pathways.

Specifically, the studies reported in this thesis are the first to suggest the importance of extracellular substrates (Matrigel, collagen, plastic) on growing EA.hy926 endothelial cells by showing that 1) EA.hy926 endothelial cells become quiescent after reaching confluence when grown on Matrigel-coated plates, but not collagen-coated or plastic plates, and 2) the presence of Matrigel decreases the sensitivity of growing cells to high concentrations of certain PUFAs (AA, EPA, and DHA). *In vitro* studies such as these provide valuable information on the response of single cell types to stimuli, however, this cellular response may not be equivalent to that of cells *in vivo*. Endothelial cell growth rate *in vitro* often exceeds cell growth *in vivo*, and culturing may result in the loss of certain cell properties while inducing the appearance of new characteristics due to the lack of a sophisticated *in vivo* environment [1]. However, thanks to this less complex

environment, it is possible to uncover endothelial cell functions that are difficult to study *in vivo.* For example, the Noble Prize in Physiology and Medicine in 1998 was awarded for the discovery of endothelium-derived relaxing factor - NO [2]. In this case, it was important to engineer the extracellular environment of endothelial cells to make it relevant to the physiological state to study their properties. Results from studies described herein have suggested the role of extracellular substrates. As a matter of fact, confluent EA.hy926 cell morphology differs visibly when they are grown on Matrigel-coated (Figure 3.1C) and plastic plates (Figure 3.1D), and Chlupac et al. [3] has shown that the substratum modulates gene expression in human saphenous endothelial cells in response to shear stress. Factors such as cell-substrate interaction and the physical properties of substrates are important in examining cellular behaviors such as survival, migration, cell cycle and differentiation [4, 5]. Additionally, factors such as shear stress, interaction with other cell types and stiffness of the cultured vessel differentially impact the endothelial cell response [6], however, examination of these is beyond the scope of this thesis.

This thesis also highlights the importance of growth state (growing, confluent) in examining the response of EA.hy926 endothelial cells. Both the effect of PUFAs on cell viability and proliferation, as well as the signaling pathways involved were growth-state dependent. With respect to the relationship between oxylipins and endothelial cells, it was shown that LOX inhibitors produced a growth-dependent effect on cell proliferation. For adherent cells such as epithelial and endothelial cells, culturing them on plates requires their growth and replication to obtain the required number of cells to perform an experiment. However, limited attention has been paid to the progression of cells growing *in vitro* until they reach confluence, especially as they relate to their responsiveness under different growth states (growing, confluent). On one hand, growing cells or replicating cells are under tight control in multicellular organism development, and mechanisms such as contact inhibition as well as other factors are known to

regulate cell cycle exit after confluence [7]. Examples of these factors include adhesion molecules such as VE-cadherin [8, 9], *Drosophila* tumor suppressor-like genes such as *dlg* and *fat* [10, 11] and their respective proteins [10], as well as contact inhibition [12, 13]. On the other hand, confluent and quiescent cells are also in an active and reversible state. Coller et al. [14] showed that confluent human diploid fibroblast cells possess unique gene expression profiles induced by contact inhibition. In terms of endothelial cells, growing cells share features with cells involved in angiogenesis, endothelium repair and plaque development. Studying confluent endothelial cell responses to different dietary stimuli is also important for providing relevant data on healthy endothelium, since investigating how growing cells respond under various conditions provides information on whether the intervention can have protective or opposite effects on endothelial cell physiology. However, growing cells cannot replace models of angiogenesis, endothelium repair and plaque development.

It is interesting that in the various studies presented in this thesis, the precursor fatty acids LA and ALA have minimal effects on cell viability and proliferation compared to the elongated fatty acids (AA, EPA and DHA) that are derived from them. LA and ALA are essential fatty acids because they cannot be synthesized in the body and have to be obtained through diet. Essential fatty acid deficiency has been documented in many pathophysiological states such as mental disorders, depression, inflammatory bowel disease, hair loss, poor immune defense, and many others [15]. LA and ALA, along with AA, GLA and DGLA which are produced from LA, and EPA and DHA which are formed from ALA, are precursors of a large family of lipid mediators, the oxylipins. Both the PUFAs and their oxygenated derivatives function as signaling molecules that play important roles in health and disease, usually by regulating various cellular and organ functions. The conjugation of PUFAs with BSA for use in treating cells is intended to mimic NEFAs in the circulation, where fatty acids are naturally bound to albumin. The neutral

effect of LA and ALA may explain why they could exist in high concentration in the form of NEFA (up 320 uM), while AA, EPA and DHA had an adverse effect even though their circulating concentrations are lower (1 uM to 14 uM) [16-18]. Although the concentration of NEFA containing C20-C22 PUFAs is low, free fatty acids released from TGs and phospholipids present in lipoproteins also contribute to the fatty acid pool that endothelial cells are exposed to via the circulation. The endothelium is also exposed to fatty acids transported in lipoproteins. For example, the DHA concentration in the phospholipid fraction of plasma can reach up to 400 μM after 1.5 g DHA is consumption daily for 6 weeks [18].

The experiments in this thesis have shown that DHA reduces the viability of confluent endothelial cells through mitochondria-dependent apoptosis, however, whether this effect is beneficial or detrimental is not easy to determine. For instance, apoptosis of endothelial cell may contribute the development of atherosclerosis by impairing the normal function of these cells in the healthy endothelium [19]. Alternatively, there may be an opportunity to employ DHA to promote apoptosis of endothelial cells for the treatment of atherosclerotic disease, since induction of endothelial cell apoptosis during cancer development is being used as a therapeutic target [20, 21]. However, it is known that apoptosis is a type of cell death that does not induce inflammation, because the cells are removed by phagocytosis and thus quickly cleared. From this point of view, apoptosis in confluent cells by DHA may be a protective mechanism, which causes less damage to the neighboring endothelium and other cell types. The concurrent decline in confluent cell proliferation in the presence of DHA may be beneficial by promoting endothelial cell quiescence or may be detrimental by hampering the regeneration of endothelial cells after a decline in cell viability. Meantime, whether there is a causal relationship between reduced cell proliferation and reduced cell viability is worth further investigation. Similarly, a

better interpretation of the reduced growing cell viability and proliferation caused by DHA also requires further studies employing models such as angiogenesis or wound repair.

Reviewing the literature as it pertains to human dietary intervention studies has shown that n-3 PUFA (EPA and/or DHA) has no effect on endothelial cell vaso-relaxation properties in healthy subjects, but improves endothelium-dependent vaso-dilation in diseased populations [22-35]. Numerous *in vitro* studies have also suggested that n-3 PUFAs are anti-inflammatory and restore normal function in both primary endothelial cells and endothelial cell lines [36, 37]. This evidence suggests that n-3 PUFAs are able to improve dysfunctional endothelial cells but provide no additional benefits to healthy endothelium in terms of their vaso-dilatory properties. Our studies, along with many others, show that various PUFAs, not only DHA [38, 39], but also LA, AA and ALA [38, 40-42] can induce apoptosis in either growing or confluent endothelial cells. Factors such as endothelial cell type, treatment time, PUFA concentration and presence or absence of extracellular substrate could contribute to the inconsistencies, however, the accumulating evidence suggests that the effects of PUFAs on endothelial cells are complex. The term PUFAs is an indication of the presence of multiple double bonds in the carbon chain structure, and these double bonds are typically linked to the adverse effects associated with non-physiologically high amounts of PUFAs. PUFAs are subject to oxidation through non-enzymatic (autoxidation and photooxidation) and enzymatic oxidation (production of oxylipins by COX, LOX and CYP). Oxidation processes destroy the double bonds, impair the functionality of PUFAs and lower their nutritional value, but also produced a vast number of oxidative products, which have effects on endothelial cells that are not fully understood. The major impacts of PUFA oxidation on endothelial cells and/or the vasculature include their production of genotoxic and cytotoxic compounds such as 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE),

which form as a consequence of oxidative stress, and promote inflammation, endothelial dysfunction, and the development of atherosclerosis [43].

Another interesting finding of this thesis that has not been previously reported was the differential and growth state-dependent activation of MAPKs (p38, ERK1/2, JNK) by DHA in EA.hy926 cells. In growing cells, p38 was activated by DHA at both early and later time points, while ERK1/2 and JNK were activated at later time points. In contrast, in confluent cells, ERK1/2 and JNK were activated by DHA at early and later time points, while only p38 was activated at early time points. A couple of studies have reported activation of MAPKs by PUFAs in endothelial cells [38, 40, 44, 45], but they did not concurrently examine the activation of p38, ERK1/2 and JNK or investigate their activation up to 8 h. Two important concepts emerge from the findings of this thesis. First, the temporal activation pattern of MAPK (p38, ERK1/2 and JNK) by DHA suggests there is cross-talk between these signaling molecules. Cells respond to a variety of extracellular stimuli through a limited number of plasma membrane embedded receptors such as GPCRs and their associated signaling pathways. Communication among different signaling pathways is a necessity and integration of their signals determines how cells respond. There is good evidence in the literature that there is a regulatory relationship between ERK1/2 and JNK [46-49] but less is known about their regulatory relationship with p38, and none of these interactions have been previously studied in the context of DHA treatment. Second, signaling transduction within a cell requires both spatial and temporal dynamics of proteins; this allows the communication of the signaling network, and determines the cellular response [50]. Also, studies have suggested that the duration of the activation of proteins results in different gene activation profiles as well as cellular responses. For example, transient activation of ERK1/2 led to cell proliferation while sustained activation led to differentiation in PC12 cells in response to nerve growth factor [51]. Like other kinase/phosphatase cascades, the

MAPK signaling cascade has many steps (MAPKKK, MAPKK, MAPK). Phosphorylation of the upstream kinase leads to activation of downstream substrates by phosphorylation, followed by de-phosphorylation of the previous kinase; this enables exit from the cascade and further transduces the signals [52]. Overall, the findings on temporal activation of MAPKs by DHA in this thesis are unique. This unique pattern may contribute to the differential effect of DHA on apoptosis in growing and confluent endothelial cells. It is also interesting that although p38 was activated by DHA in both growth states, only its activation in confluent cells has a regulatory relationship with caspase-3, which leads to apoptosis. This evidence further emphasizes the importance of growth state in the cellular responses to DHA and its mechanisms of action. Future studies on this activation pattern and the resulting signaling transduction are needed to further delineate how MAPK signaling contributes to the biological effects of DHA in endothelial cells.

The last section of this thesis examined the role of oxylipins, specifically those generated via LOX, on endothelial cell viability and proliferation. The experimental approach taken was to inhibit individual LOX enzymes using pharmaceutical compounds. An examination of the literature indicated that inhibition of 5-LOX by AA-861, MK-886 and NDGA decreased the proliferation rate of human pulmonary aortic endothelial cells [53]. However, it is noted the investigators did not examine 5-LOX products or 5-LOX activity to verify that the observed effects were attributable to 5-LOX. In the current thesis, the surprising finding was that PD146176, which is claimed to be a specific 15-LOX inhibitor with a low IC_{50} [54], did not inhibit 15-LOX in EA.hy926 endothelial cells. There is confidence in this interpretation since an examination of the oxylipin profile after the inhibitor treatment showed that there was no reduction of 15-LOX products. This is the first report questioning the selectivity of this inhibitor. Other studies that used PD146176 in either a rabbit model or with BAECs did not validate

PD146176 as a 15-LOX specific inhibitor by examining the production of 15-LOX oxylipins [54-56]. However, PD146176 was originally found to inhibit rabbit reticulocyte 15-LOX activity [54]. Cell type specificity as well as concentration may be the reason this inhibitor is not working the same way, but further assessment will be necessary to address this point.

Although the negative effects of PD146176 on EA.hy926 endothelial cells were not due to its inhibitory effect on 15-LOX, 15-LOX products have been documented in the regulation of endothelial cell physiology with inconsistent results. In particular, there is limited data regarding their effects on cell viability and proliferation. 13-HpODE but not 13-HODE induced bovine mammary endothelial cells apoptosis and impaired barrier function [57]. 13-HpODE was also reported to increase eNOS expression in BAECs [58] and increase ICAM-1 expression in HUVEC [59]. 15-HpETE was reported to induce BAEC apoptosis [56], while 15-HETE was found to protect bovine pulmonary aortic endothelial cells from apoptosis under hypoxic conditions [60]. Future studies using selective 15-LOX inhibitors (chemical or genetic) are therefore warranted to examine the role of 15-LOX in endothelial cell physiology.

On the other hand, various studies have suggested that 15-LOX is not detectable under basal conditions, only in IL-4 induced endothelial cells [61, 62]. PD146176 was shown to reduce 15-HETE, 17-HDHA and NPD1 production in human primary retinal pigment epithelial cells preconditioned by 50 μM H_2O_2 for 6 h [63]. PD146176 also reduced 15-HETE production in human bronchial epithelial cells [64]. The inability of PD146176 to affect 15-LOX in our study might due to the distinct active state of 15-LOX or the growth state of endothelial cells, which indicated further investigation is warranted.

It is important to examine the oxylipin profile in any study of oxylipin producing enzymes. On the one hand, detecting representative oxylipins from a specific enzyme is not equivalent to determining the activity of the enzyme. For example, 15-HETE and 13-HODE are produced

from AA and LA, respectively, through 15-LOX, but they can also be produced from COX under certain experimental conditions in endothelial cells [65, 66]. On the other hand, the biological actions of oxylipins may not be dependent upon an individual compound, but instead be the result of their combined effects as a group. Thus, the effect of individual oxylipins *in vitro* may not be representative of the effect *in vivo.* Their relative amount in the system may also be important in determining the overall effect. For example, PGE_3 derived from EPA is more potent in increasing the permeability of the HUVEC monolayer than PGE_2 derived from AA [67]. Likewise, LTB_5 from EPA has no significant effect on PMN cell adhesion to ECs, while LtB_4 from AA significantly increases PMN cell adhesion to EC [68]. This may explain why EPA supplementation induces an anti-inflammatory effect as EPA and AA may compete for the same enzyme - LOX - leading to greater production of LTB_5 from EPA instead of LTB_4 from AA, with the latter being more pro-inflammatory.

Experiments addressing the potential molecular mechanism by which PD146176 affects endothelial cells showed that treatment with the inhibitor alone induced activation of p38 MAPK and PPARα after 30 min and 8 h in both growing and confluent cells. This coincides with the observations that 1) DHA activated p38 MAPK at early time points and 8 h in growing cells, and at early time points in confluent cells (Figure 4.3), and 2) DHA induced activation of PPARα after 8 h treatment in growing cells (Figure A.3). Since the action of PD146176 on endothelial cells was associated with a change of CYP products, it appears that CYP oxylipins and DHA may operate via the same signaling pathways, presumably, p38 MAPK and PPARα. More interestingly, treatment with DHA (Figure A.3) or PD146176 (Figure 5.2, Figure 5.3) alone activated PPARα at 8 h in growing cells, but combined treatment of DHA and PD146176 resulted in a reduction of PPARα at 8 h compared to PD146176 treatment alone (Appendix Figure A.4). This gives confidence to hypothesize that a sequential relationship exists between

DHA, CYP oxylipins and PPARα activation. Future studies addressing this hypothesis could lead to the discovery of PPARα agonists/antagonists relevant to the roles of oxylipins and DHA in disease processes, as well as a better understanding of the mechanisms of action of oxylipins and PUFAs in the modulation of endothelial cell functions.

The previous paragraphs provide linkages from the experimental results to the various sub-hypotheses that formed the basis of this thesis. Overall, the results suggest that 1) dietary PUFAs (LA, AA, ALA, EPA and DHA) can affect the viability and proliferation of EA.hy926 human endothelial cells, and the modulation of PUFAs on cells is growth state (growing, confluent)-dependent and substrate-dependent as well as PUFA specific; 2) DHA modulates endothelial cell apoptosis through activation of caspase-3 and MAPK in a growth-dependent manner; and 3) changes in oxylipin production (resulting from application of PD146176), especially CYP products, is related to changes in cell viability and proliferation which are growth state-dependent.

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Chapter 7: Conclusions and Implications

7.1 Conclusions

The current thesis was designed to investigate the effects of dietary PUFAs, including LA, AA, ALA, EPA and DHA, and LOX-derived oxylipins (by using specific LOX inhibitors) on the viability and proliferation of growing and confluent (quiescent) EA.hy926 human endothelial cells. This thesis also examined the role of MAPK (p38, ERK, JNK) signaling in mediating these effects.

Characterization of the growth of EA.hy926 endothelial cells grown on different substrates such as Matrigel, collagen and plastic surface, initially showed that cells became quiescent after reaching confluence when they were grown on Matrigel-coated plates, but not collagen-coated or plastic surfaces. Subsequently, it was found that the response of EA.hy926 endothelial cells to dietary PUFAs was fatty acid specific, as well as substrate- and growth state-dependent. Specifically, it was observed that AA, EPA and DHA, but not their precursor fatty acids LA and ALA, negatively affected the viability and proliferation of both growing and confluent cells. Furthermore, DHA was shown to have the most potent effect. Substrate was also identified as having a role in the cell response to PUFAs, and that cells grown on Matrigel-coated plates were more resistant to high concentrations of AA, EPA, and DHA than cells growing on plastic plates. PUFAs affected growing and confluent EA.hy926 endothelial cells in a different manner when compared to cells grown on plastic plates, and confluent cells were more resistant to high concentrations of AA, EPA and DHA than growing cells.

This thesis revealed the important role of growth state for the EA.hy926 endothelial cell response to PUFA by showing that DHA induced apoptosis of confluent but not growing cells

through a caspase-3 and p38 MAPK-dependent pathway. Results showed that MAPKs including p38 MAPK, ERK1/2 and JNK were activated by DHA in a growth state-dependent manner. A unique pattern of p38 MAPK activation by DHA compared to other PUFAs (LA, AA, ALA, and EPA) was also observed, which led to the identification of a temporal sequence of activation of p38 MAPK, ERK1/2 and JNK in response to DHA. This may suggest a hierarchy of MAPKs in their signaling and cross-talk, which warrants further investigation.

This investigation was the first to report that compound PD146176 does not function as a 15-LOX inhibitor, but instead inhibits CYP activity in EA.hy926 endothelial cells. The reduction of anti-inflammatory and pro-angiogenic oxylipins such as 5,6-, 8,9-, and 11,12-EpETrE may be responsible for the decrease in the viability and proliferation of cells, and may contribute to increased activation of inflammation associated with p38 MAPK phosphorylation and to de-activation of the cell survival kinase Akt after PD146176 treatment. However, their specific function warrants further investigation.

To conclude, this thesis suggests the extracellular substrates and growth state have important roles in the EA.hy926 human endothelial cell response to PUFAs and oxylipins in terms of viability and proliferation. Examining the molecular mechanisms also confirmed that regulation of endothelial cells by PUFAs was through a growth state-dependent activation of MAPKs. It has also been possible to conclude that the commercial 15-LOX inhibitor PD146176 does not inhibit 15-LOX in EA.hy926 endothelial cells, but rather targets CYP. These findings suggest CYP oxylipin products have a major role in modulating endothelial cell viability and proliferation.

7.2 Implications

Based on the studies presented in this thesis, which identified the role of extracellular substrates on the EA.hy926 endothelial cell response to PUFAs, it is recommended that future studies on endothelial cells or other cell types consider characterizing cell growth with appropriate substrates before treatment. The extracellular matrix appears to be an essential factor for obtaining the physiological response of endothelial cells to dietary factors.

The studies reported in this thesis also suggested that the growth state of EA.hy926 cells contributes significantly to the cellular response to PUFAs. Although the growing cells used in this study were not identical to either repairing cells or cells involved in angiogenesis *in vivo*, their growth properties were different from those of confluent (quiescent) cells. Different cellular responses in different growth states highlights the importance of considering growth state of endothelial cells when examining the effect of dietary factors. Studies on dietary supplementation of PUFAs may also need to distinguish the possible differential effects in populations with healthy or activated/dysfunctional endothelial cells given the fact that many dietary intervention studies of n-3 PUFAs showed no effect in healthy or younger populations.

The observation of DHA-induced apoptosis in confluent but not growing cells suggested that DHA and other dietary factors might modify endothelial cell survival. However, the role of endothelial cell apoptosis and its regulation by DHA still warrants further investigation, particularly as it may relate to differential effects of DHA in healthy versus dysfunctional endothelium. The growth conditions of endothelial cells may also have an association with the efficacy of PUFAs. The results obtained with PD146176 suggest that developing specific inhibitors of LOX is urgent and interpretation of the results on experiments with inhibitors needs to proceed with caution. Further study of the effect of oxylipins individually and in combination are needed to understand the complex interplay on endothelial cells.

Chapter 8: Strengths and Limitations

8.1 Strengths

- The EA.hy926 endothelial cell line is a widely used and robust endothelial cell line that is easy to culture.
- Matrigel contains a mixture of ECM components, and provides a physiologically relevant environment that mimics the basement membrane that endothelial cells normally grow on.
- The question of whether confluent EA.hy926 endothelial cell were quiescent when they were grown on different substrates was addressed.
- The availability of highly purified PUFAs (LA, AA, ALA, EPA and DHA) employed in this study enabled an examination of the effect of single PUFAs.
- The method of combining PUFA with 3% BSA represents the non-esterified form of fatty acids present in the bloodstream.
- Appropriate methodological approaches, including cell-based assays, Western blotting, and LC/MS/MS, were used.
- The effects of PUFAs in EA.hy926 endothelial cells were compared in both growing and confluent states.
- Dose and time dependence of PUFA treatments on viability and proliferation were performed.
- A systematic study of MAPKs (p38 MAPK, ERK1/2 and JNK) was completed, and the temporal sequence for activation of MAPKs was investigated for up to 8 hours.

- p38 MAPK activation by DHA was investigated in relation to the upstream kinase (MKK3/MKK6) and the phosphatase (DUSP1) which regulate p38 MAPK.
- Apoptosis was confirmed by a histological assay and by showing evidence of caspase-3 activation.
- Whether apoptosis mediated by DHA was via the intrinsic or extrinsic pathway was investigated.
- Cross-talk among p38, Akt and PPARα was established in endothelial cells.
- Dose and time dependence of PD146176 treatment on the responses of p38, Akt and PPARα were determined.
- The inhibition (or lack thereof) of LOX by the compound PD146176 was determined by examining oxylipin profiles.

8.2 Limitations

- The EA.hy926 endothelial cell line is derived from HUVEC; although it is widely used to study endothelial cell properties, it is not equivalent to primary endothelial cells or other cell lines such as HMEC-1.
- Subconfluent endothelial cells grown in culture do not possess the complete characteristics of growing/repairing endothelial cells *in vivo*, such as the lack of contact with other cell types and the absence of flow.
- Single PUFA effects on EA.hy926 endothelial cells may not be equivalent to the effect of the PUFA *in vivo*, because endothelial cells would be interacting with a composite of lipid families.
- The specificity of chemical inhibitors cannot be guaranteed as they may have unknown effects on other signaling pathways.
- Lack of specific LOX inhibitors hampers the study of the effect of specific LOX products on endothelial cell physiology.

Chapter 9: Future Directions

- Investigate the effect of PUFAs on primary endothelial cells and other endothelial cell lines.
- Investigate the death pathway in growing endothelial cells treated with DHA.
- Investigate which p38 MAPK isoform(s) is/are activated with DHA treatment in growing and confluent endothelial cells.
- Investigate the cross-talk amongst p38 MAPK, ERK1/2 and JNK in DHA-treated EA.hy926 endothelial cells.
- Investigate the effect of inhibiting the caspase cascade on confluent cell apoptosis after DHA treatment.
- Consider using a genetic approach rather than pharmaceuticals to inhibit enzymes responsible for generating oxylipins.
- Investigate the effect of CYP inhibitors on endothelial cell viability and proliferation.
- Investigate whether adding back the oxylipins that are reduced with PD146176 treatment can rescue cell viability and proliferation.
- Investigate EA.hy926 endothelial cell growth on other substrates such as gelatin, fibronectin, elastin, laminin, etc. and combinations of substrates.
- Investigate the effect of PUFAs (LA, AA, ALA, EPA, DHA) on other functions of endothelial cells such as migration, barrier function, angiogenesis, lipid droplet formation, etc.
- Investigate the effect of FA combinations that represent the fat composition in different diets such as Western diet, Mediterranean diet, etc. on endothelial cell function.

- Compare the effects of DHA provided as a FFA conjugated to BSA with liposomes containing phospholipids enriched with DHA on endothelial cell function.
- Investigate the effect of n-6 PUFA and n-3 PUFA combinations in different ratios on endothelial cell function.
- Investigate which receptor(s) is/are involved in mediating the effects of DHA on confluent cells.
- Investigate the role of DUSP members other than DUSP1 in the de-phosphorylation of p38 MAPK after DHA treatment.
- Investigate the oxylipin profile of confluent endothelial cells.treated with DHA
- Investigate the effect of DHA and LOX inhibitors on endothelial cell function.
- Investigate the oxylipin profile in confluent endothelial cells after PD146176 treatment.

Appendix

Appendix A

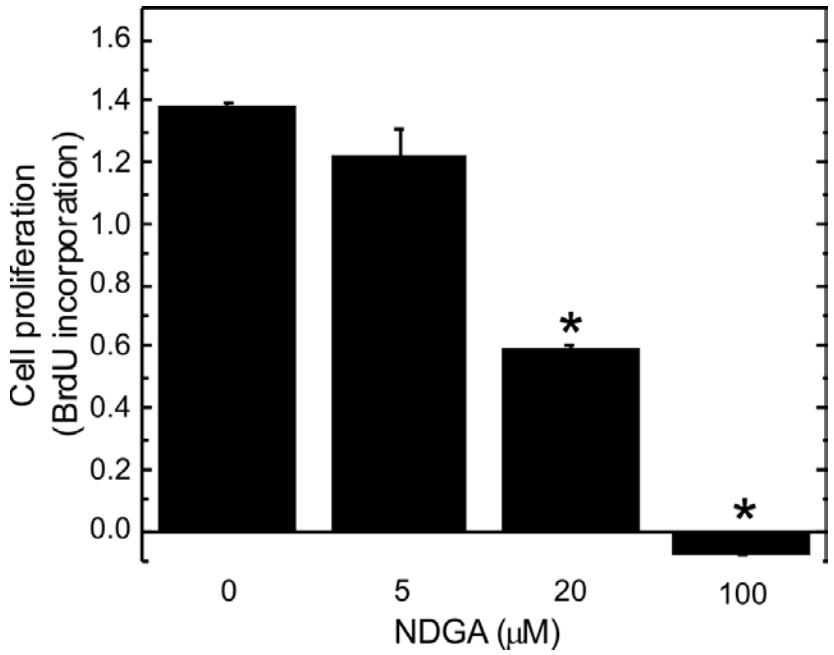


Figure A.1 Effects of different concentrations of NDGA on cell proliferation of confluent cells.

Confluent EA.hy926 endothelial cells were treated with NDGA (5-LOX inhibitor) at 0, 5, 20, and 100 μM for 24 h before measuring cell proliferation by BrdU assay. Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 μM (vehicle control).

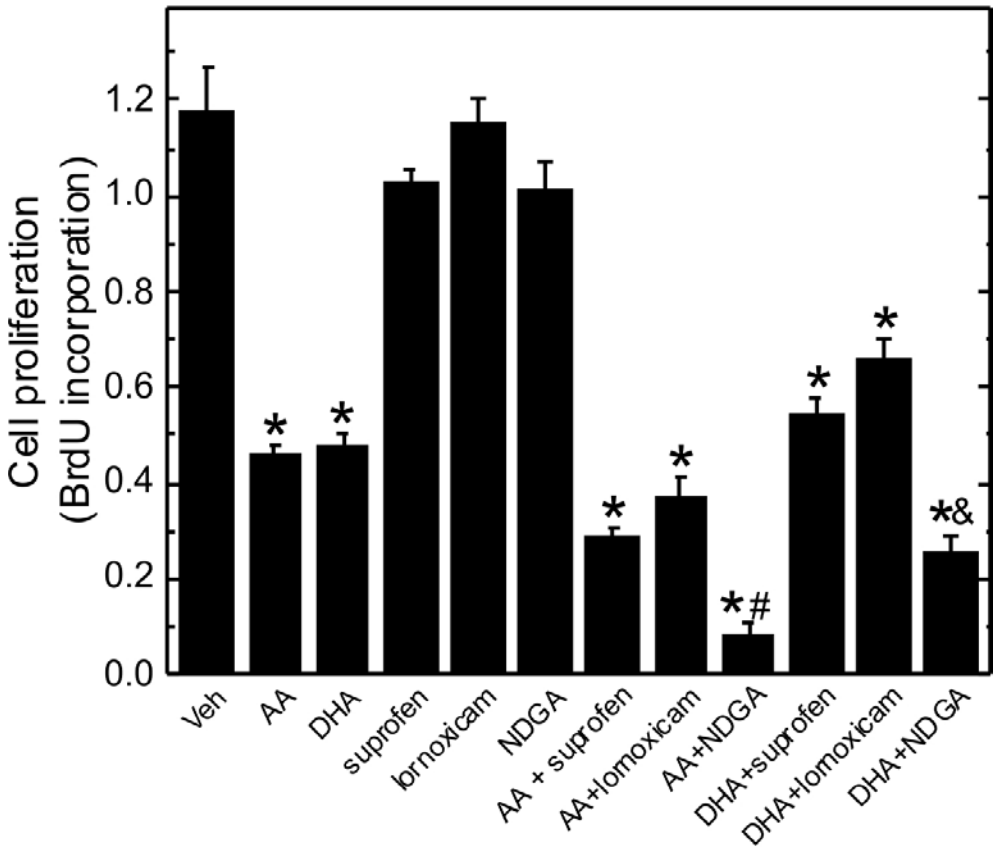


Figure A.2 Effects of LOX inhibitors alone and combined with AA/DHA on cell proliferation in confluent endothelial cells.

Confluent EA.hy926 endothelial cells were treated with AA, DHA, suprofen, lornoxicam and NDGA alone or in various combinations for 24 h before performing measuring cell proliferation by BrdU assay. The concentrations of AA, DHA, suprofen, lornoxicam, NDGA used were 40 μM, 80 μM, 25μM, 0.1μM and 5 μM, respectively. Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 μM (Vehicle control). $^{\#}$Significantly different ($p<0.05$) from AA. $^{\&}$Significantly different ($p<0.05$) from DHA.

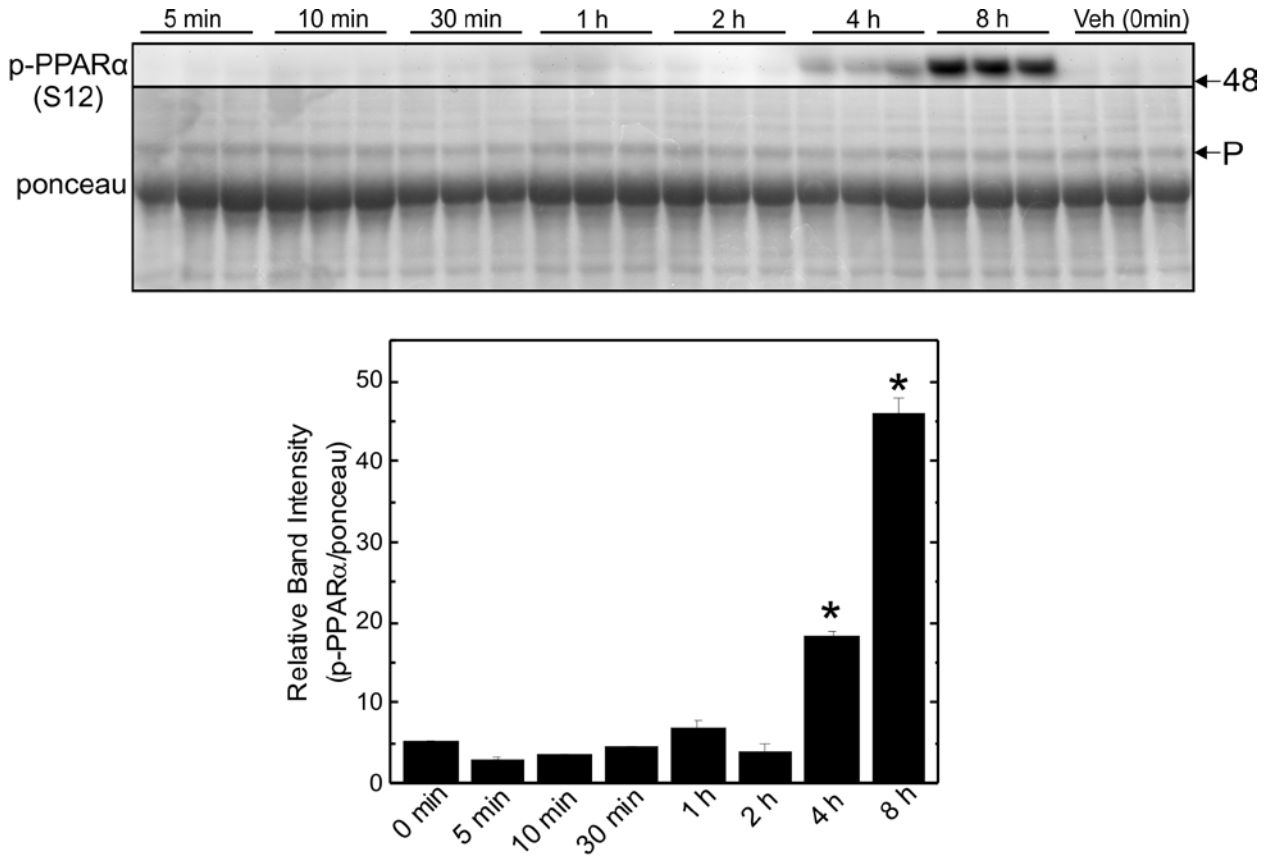


Figure A.3 Examination of PPARα activation with DHA treatment of growing cells.

Growing EA.hy926 endothelial cells were treated with 125 μM DHA for various times (0 min - 8 h). Levels of p-PPARα were determined by Western blotting of cell lysates. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in upper Panel. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. Veh - vehicle.

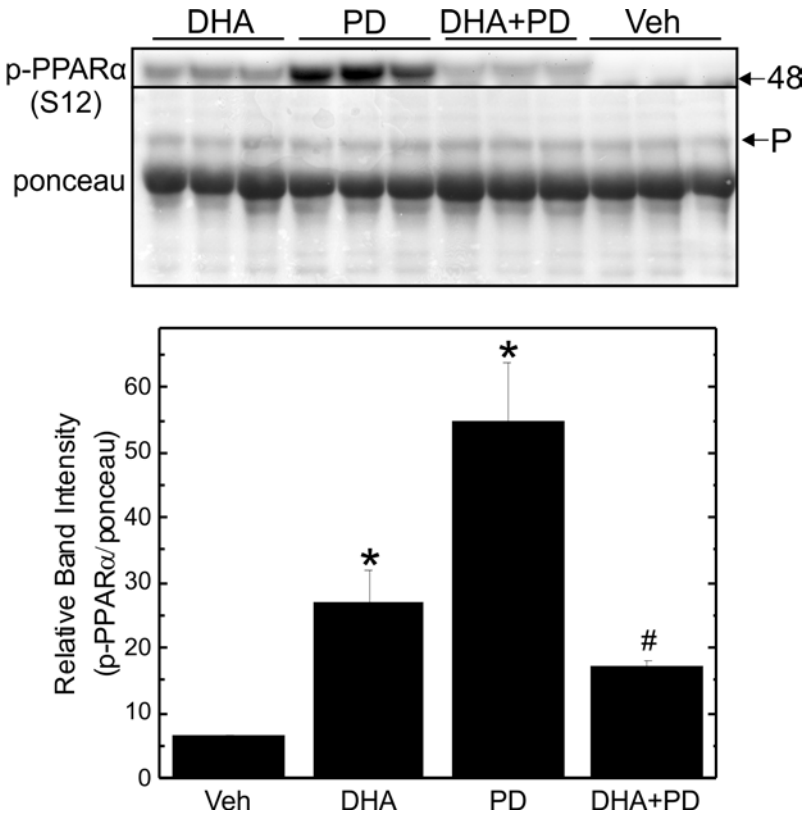


Figure A.4 Examination of PPARα activation with DHA and PD146176, alone and in combination, in growing cells.

Growing EA.hy926 endothelial cells were treated individually with DHA and PD, as well as in combination for 8 h. Levels of p-PPARα were determined by Western blotting of cell lysates. Concentrations of DHA and PD used were 125 μM and 20 μM, respectively. PD was added to the cells 30 min before adding DHA in the combined treatment. Representative blots showing 3 lanes per treatment with molecular weight markers indicated (arrow, mass in kDa) are presented in the upper Panel. Densitometry was used to quantify the intensity of the bands and data were normalized to a band visualized by Ponceau S staining (arrow labeled P on image). Data are presented as means ± SEM (n=3). *Significantly different ($p<0.05$) from 0 min. $^{\#}$Significantly different ($p<0.05$) from PD. PD - PD146176, Veh - vehicle.