

MOLECULAR MECHANISM OF ANANDAMIDE-INDUCED APOPTOSIS IN NON-MELANOMA SKIN CANCER

By

Eman Soliman

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Director of Dissertation: Dr. Rukiyah Van Dross

Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University

Non-melanoma skin cancer (NMSC) is the most common cancer in the United States. The absence of selective toxicity is the major problem associated with chemotherapeutic and radiation therapy for NMSCs. The goal of the present study was to determine the molecular mechanism of action of a potential selective treatment for NMSC, arachidonoyl ethanolamide [AEA, (also known as anandamide)]. Our research revealed that two factors provide AEA with its selective toxicity, the elevated cyclooxygenase-2 (COX-2) levels and the activated endoplasmic reticulum (ER) stress signaling in tumorigenic keratinocytes which differentiates them from normal cells. COX-2 is an enzyme that promotes tumor development by synthesizing E-series prostaglandins (PGs) that promote cell proliferation. Selective inhibition of COX-2 is successfully used in treatment of skin cancer. Rather than inhibiting COX-2, the novel approach of exploiting the overexpression of COX-2 was utilized. In tumorigenic keratinocytes, AEA was metabolized by COX-2 to cytotoxic J-series prostaglandin-ethanolamides (PGJ₂-EAs) that are

structurally distinct from arachidonic acid (AA)-derived J-series PGs. This data is novel and to the candidate's knowledge, this is the first report showing that PGJ₂-EAs are produced as an AEA metabolic product. Furthermore, several studies showed that J-series PGs derived from AA induce oxidative stress, ER stress and apoptosis. Therefore, the candidate hypothesized that AEA is metabolized to PGJ₂-EAs, which induce oxidative stress followed by ER stress and ultimately apoptosis as a novel mechanism of action of AEA. The candidate also took advantage of evidence, which shows that ER stress is moderately elevated in tumor cells when compared to normal cells. Under these circumstances, the exposure of cells to agents that induce additional stress is expected to cause overwhelming ER stress and subsequently apoptosis selectively in tumor cells. Indeed, the candidate determined that AEA induced ER stress-mediated apoptosis in COX-2 expressing tumor cells but not in normal cells. Hence, the present study provides an alternative strategy for topical treatment of NMSCs that will be selectively cytotoxic to cancer cells but not detrimental to normal, healthy surrounding cells.

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MELANOMA SKIN CANCER**

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By

Eman Soliman

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Eman Salah Abd El-Aziz Soliman

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By

Eman Soliman

APPROVED BY:

DIRECTOR OF
DISSERTATION: _____

(Rukiyah Van Dross-Anderson, PhD)

COMMITTEE MEMBER: _____

(Abdel A. Abdel-Rahman, PhD)

COMMITTEE MEMBER: _____

(Brett D. Keiper, PhD)

COMMITTEE MEMBER: _____

(Ethan J. Anderson, PhD)

COMMITTEE MEMBER: _____

(Saame Raza Shaikh, PhD)

CHAIR OF THE DEPARTMENT
OF (Pharmacology and Toxicology): _____

(David Taylor, PhD)

DEAN OF THE
GRADUATE SCHOOL: _____

Paul J. Gemperline, PhD

This work is dedicated to my lovely parents. The happiest moments of my life have been the few which I have passed in the bosom of you. Your inspiration, encouragement and prayers are the keys to my success.

AND

To my husband, Ahmed Elhassanny, who by all accounts earned this degree with me. Thank you for your advice, love, wisdom and support. To my loving boys, Mohamed, Omar, and Eyaad. I spent a long time away from you in the past five years of my life. Thank you ALL for your understanding and patience. Without your love, I would not have been able to reach my dreams.

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LIST OF SYMBOLS OR ABBREVIATIONS

15d-PGJ ₂	15 deoxy $\Delta^{12,14}$ Prostaglandin J2
15d-PGJ ₂ -EA	15 deoxy $\Delta^{12,14}$ Prostaglandin J2-ethanolamide ($\Delta^{12,14}$ prostamide J2)
AA	Arachidonic acid
AARE	Amino acid responsive element
AEA	Arachidonoyl-ethanolamide (also known as Anandamide)
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carboxamide
AM630	1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindole
AMT	Anandamide membrane transporter
ANOVA	Analysis of Variance
ATF	Activating transcription factor
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BiP/GRP78	Binding immunoglobulin protein/ 78 kDa glucose-regulated protein
C/EBP	CCAAT/Enhancer-Binding Protein
cAMP	Cyclic adenosine monophosphate
CBR	Cannabinoid receptor
CHOP10	CCAAT/Enhancer-Binding Protein (C/EBP) Homologous Protein-10
CID	Collision-induced dissociation
CM-H ₂ DCFDA	Chloromethyl-2',7' dichlorodihydrofluorescein diacetate
COX-2	Cyclooxygenase-2
DR5	Death receptor 5

DTNP	2,2'-Dithiobis(5-nitropyridine)
ECS	Endocannabinoid system
EDTA	Ethylenediaminetetraacetic acid
EET-EA	Epoxyeicosatrienoic acids ethanolamide
EGTA	Ethylene glycol tetraacetic acid
eIF2 α	Eukaryotic initiation factor -2 alpha
ELISA	Enzyme linked immunosorbant analysis
ER	Endoplasmic reticulum
ERAD	ER associated degradation
Ero1	ER oxidoreductase 1
ERSE	ER stress responsive element
ESI	Electrospray Ionization
ETC	Electron transport chain
ETOH	Ethanol
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
GADD	Growth arrest and DNA damage-inducible protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
H ₂ O ₂	Hydrogen peroxide
HAEA	Hydroxy anandamide
HETE-EA	Hydroxyeicosatetraenoic acid ethanolamide
HNE	4-Hydroxy-2-nonenal

H-PGDS	Hematopoietic prostaglandin D synthase
HSP-70	Heat shock protein-70
IRE1	Inositol requiring kinase-1
LC-MS	Liquid chromatography-mass spectroscopy
LOX	Lipoxygenase
L-PGDS	Lipocalin prostaglandin D synthase
m/z	Mass to charge ratio
MMLV	Moloney Murine Leukemia Virus
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NMSC	Non-melanoma skin cancer
PBS	Phosphate buffer saline
PDI	Protein disulfide isomerase
P-eIF2 α	Phosphorylated eIF2 α
PERK	Double-stranded RNA-activated protein kinase (PKR)-like Endoplasmic Reticulum kinase
PG	Prostaglandin
PGJ ₂ -EAs	J-series PG-ethanolamides (J series prostamides)
P-IRE1	Phosphorylated IRE1
PP1	Protein phosphatase 1
PPAR	Peroxisome proliferator-activated receptor

P-PERK	Phosphorylated PERK
RLU	Relative luminescence units
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SEM	Standard error of mean
SPE	Solid phase extraction
TEE	Tris- EDTA- EGTA
THC	Tetrahydrocannabinol
t-IRE1	Total IRE1
TRITC	Tetramethylrhodamine isothiocyanate
TRPV1	Transient potential vanilloid receptor
UPR	Unfolded protein response
UV	Ultra violet
XBP1	X-box binding protein1
Δ^9 THC	Δ^9 tetrahydrocannabinol

CHAPTER ONE: GENERAL INTRODUCTION

1.1. Cancer

Cancer is a disease that is characterized by uncontrolled proliferation of abnormal cells. Cancer typically develops as a consequence of genetic and epigenetic alterations of genes that regulate cell growth, cell death and DNA repair. The first stage in tumor development is initiation, during which irreversible DNA damage occurs in the genetic sequence of tumor promoter proteins or oncogenes. In the second stage of tumorigenesis, promotion, continuous exposure to agents that promote proliferation and/or inflammation causes clonal expansion of the initiated cell and more extensive DNA damage. In the final and irreversible stage of tumor formation, progression, cells transition from benign to malignant tumors and exhibit autonomous growth as well as the ability to metastasize. These events culminate in the formation of tumors which are diagnosed in more than 500,000 individuals each year (Weinburg, 2013).

1.1.1. Non Melanoma Skin Cancer

Skin cancer is the most common cancer in the United States. There are two major categories of skin cancer: melanoma, which originates from melanocytes, and non-melanoma skin cancer (NMSC) which originates from either squamous cells (squamous cell carcinoma) or basal cells (basal cell carcinoma) (Madan et al., 2010). Non melanoma skin cancers (NMSCs) comprise the most frequently occurring forms of skin cancer. According to the skin cancer foundation's most recent estimates, "More than two million people in the US are developing over 3.5 million NMSCs every year" (Skin Cancer Foundation, 2012). Although death from these cancers is uncommon, NMSCs are associated with substantial annual health care costs. The estimated cost in 2004 was \$1.4 billion direct costs and \$76.8 million indirect costs (Bickers et al., 2006; Guy and Ekwueme, 2011). The incidence of NMSCs has been increasing rapidly in the

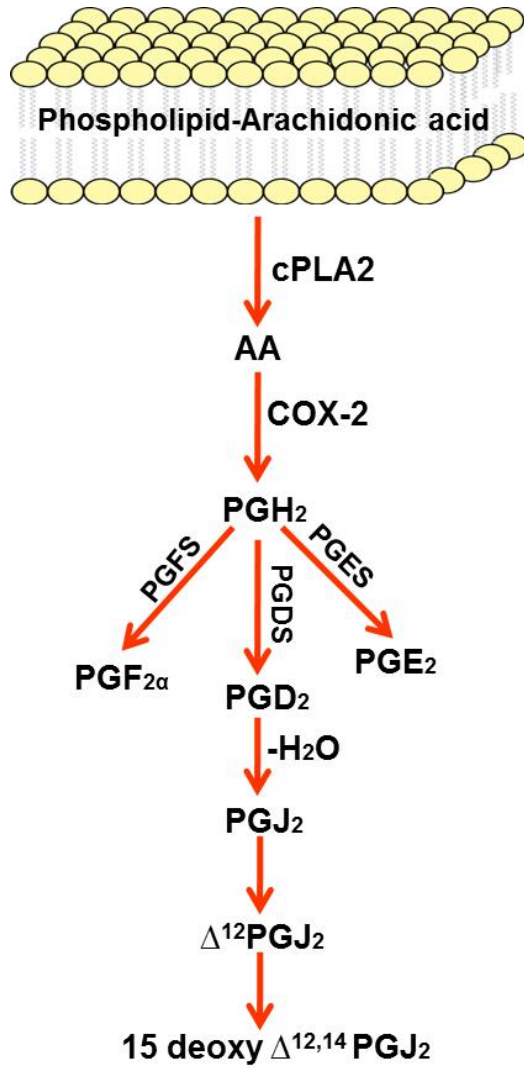
past few decades probably due to increased sun and UV light exposure, increased outdoors activities, and/or ozone depletion (Gloster, Jr. and Brodland, 1996;Armstrong and Kricger, 2001;Diepgen and Mahler, 2002).

1.2. Role of cyclooxygenase-2 (COX-2) in NMSCs

It has been reported that UV irradiation induces COX-2 expression in the skin (Fischer et al., 1999). COX-2 is the rate-limiting enzyme for the production of prostaglandins (PGs) from arachidonic acid (AA) (Figure 1.1) (Pentland, 1998). AA is liberated from membrane phospholipids by the enzyme phospholipase A2. COX-2 then oxygenates AA to PGH₂ followed by its metabolism by PG synthase-E, -F_{2α}, or -D to PGE₂, PGF_{2α}, and PGD₂, respectively. PGD₂ is then dehydrated to J-series PGs. Increasing evidence points to a role for COX-2 and its products, notably PGE₂, in the development of NMSCs. Compared to normal keratinocytes, NMSCs typically overexpress COX-2, with elevated levels of COX-2 correlating with the severity of skin tumors (Buckman et al., 1998;Athar et al., 2000;Spallone et al., 2011). COX-2 and PGE₂ promote keratinocyte proliferation, angiogenesis, and cell migration by increasing the expression of oncogenes, cytokines and growth factors (Rundhaug et al., 2007;Rundhaug et al., 2011).

Figure 1.1: Arachidonic acid metabolism by COX-2

AA is liberated from membrane phospholipids by cytosolic phospholipase A2. COX-2 then oxygenates AA to PGH₂ followed by its metabolism by PG synthase-E, -F₂α, or -D to PGE₂, PGF₂α, and PGD₂, respectively. PGD₂ is then dehydrated to J-series PGs (PGJ₂, Δ^{12,14} Prostaglandin J₂ and 15 deoxy Δ^{12,14} Prostaglandin J₂).



1.3. Anti-tumorigenic effect of cyclopentenone J-series prostaglandins

Contrary to the pro-proliferative E-type PGs, D-type and J-type PGs induce cell death. Three J-series prostaglandins exist (i) PGJ₂, (ii) Δ^{12} PGJ₂ and (iii) 15-deoxy $\Delta^{12,14}$ PGJ₂ (15d-PGJ₂) (see figure 1.1). Previous studies in our lab and others revealed that J-series PGs induce apoptosis (Clay et al., 1999; Van Dross, 2009; Wang and Mak, 2011; Kuc et al., 2012). In contrast, Millan et al. found that DMBA/TPA-mediated skin tumorigenesis was enhanced when 15d-PGJ₂ was applied topically during the tumor initiation phase (Millan et al., 2006). This finding was likely due to the concentration of J-series PGs used in the study as it has been found that low concentrations of 15d-PGJ₂ are cytoprotective while high concentrations are pro-apoptotic (Levonen et al., 2001).

Numerous studies have determined that J-series PG-mediated apoptosis is regulated by its ability to promote reactive oxygen species (ROS) production (Kondo et al., 2001; Chen et al., 2005). The unique receptor-independent biological effects of the J-series PGs are proposed to be due to the presence of a highly reactive α , β -unsaturated carbonyl group in the cyclopentenone ring that can covalently interact via Michael addition reaction with nucleophiles such as free sulfhydryls of cysteine residues in cellular proteins. J-series PGs induce oxidative stress by covalently binding to sulfhydryl-containing glutathione (GSH) and other proteins that regulate cellular redox status (Kondo et al., 2001; Uchida and Shibata, 2008). In addition, J-series PG-induced oxidative stress is also mediated by an increase in reactive molecules including 4-hydroxy-2-nonenal (HNE) and acrolein (Kondo et al., 2001).

1.4. Reactive oxygen species (ROS)

Reactive oxygen species are reactive molecules containing oxygen such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH[•]). In eukaryotic cells, ROS can

be generated by many organelles including mitochondria and endoplasmic reticulum (ER). Mitochondria are the major source of ROS in the cell. During oxidative phosphorylation in the mitochondria, a small fraction of electrons from the electron transport chain (ETC) are transferred to O₂ to generate superoxide anion which then can give rise to other ROS (Cadenas and Davies, 2000). The endoplasmic reticulum is also considered an important source of ROS in the cell. During oxidative protein folding, ER oxidoreductase 1 (Ero1) accepts electrons from protein disulfide isomerase (PDI) and transfers them to molecular oxygen (O₂) to produce H₂O₂, the major ROS produced in the ER lumen (Cao and Kaufman, 2014). ROS are second messengers in signal transduction. A low concentration of ROS is required for many physiological functions such as immune defense against pathogens, induction of mitogenic response and maintaining cellular homeostasis (Finkel, 1998; Droge, 2002). Under normal conditions, the ROS concentration is maintained within strict physiological limits by enzymatic and non-enzymatic antioxidant defense systems. The enzymatic components of antioxidant system include glutathione reductase, glutathione peroxidase, catalase, superoxide dismutase, etc (Mates et al., 2012). The most important endogenous non-enzyme antioxidant is Glutathione (GSH). GSH is a thiol containing compound, which is abundant in cytosol, nucleus and mitochondria and maintains the intracellular redox balance. GSH is a cofactor for detoxifying enzymes such as GSH peroxidase and GSH transferase. GSH also acts as a scavenger of HO· and singlet oxygen and detoxifies H₂O₂ and lipid peroxides (Masella et al., 2005; Kaur et al., 2014). Tipping the balance towards prooxidants favors the production of ROS and oxidative stress. High concentrations of ROS cause oxidative damage to cellular proteins, lipids and DNA (Kaur et al., 2014).

1.4.1. Oxidative stress and cancer

Cancer cells are characterized by an altered redox environment. They produce higher levels of ROS compared to normal cells. Relatively high ROS production in cancer cells activates signaling pathways that regulate cell proliferation, survival, angiogenesis and metastasis (Nourazarian et al., 2014). However, excessive levels of ROS induce cell cycle arrest and cell death. Hence, this unique redox status of cancer cells can be exploited to selectively target cancer cells. Cancer can be treated by either dampening ROS levels below the level, which is required for cell proliferation or by promoting ROS production and, therefore, causing lethal oxidative damage (Glasauer and Chandel, 2014).

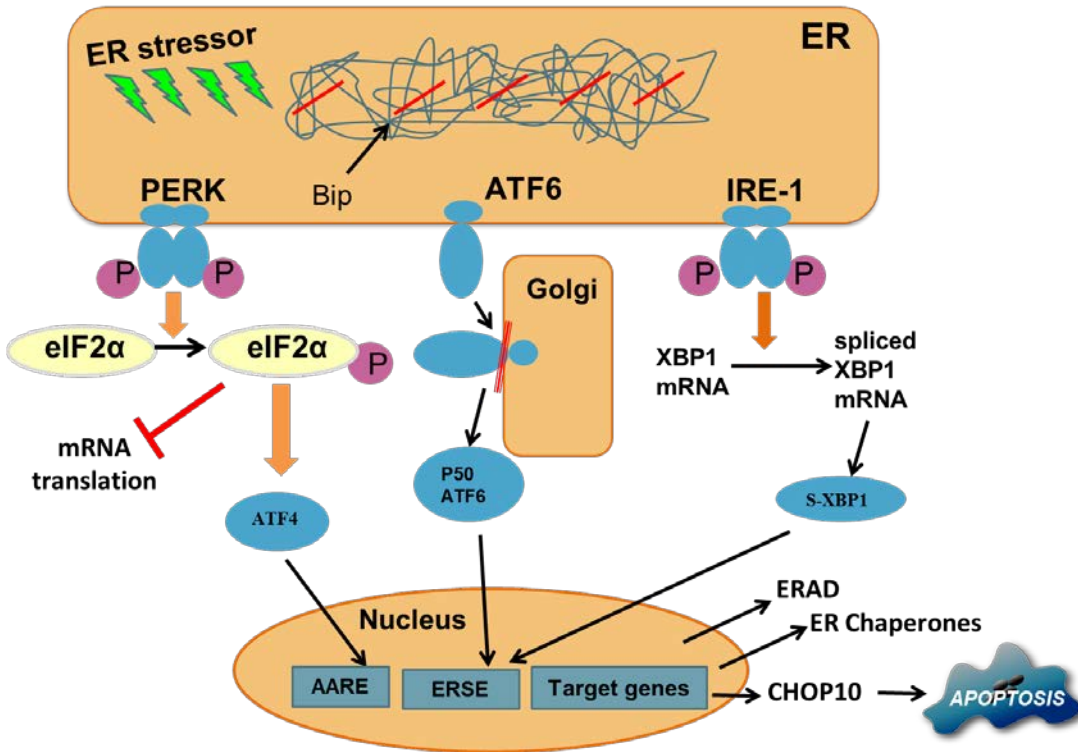
1.5. Endoplasmic reticulum stress

ER stress occurs when the cell's capacity to fold proteins is exceeded by the protein folding load. ER stress results in dysregulation of protein folding, accumulation of unfolded proteins and activation of the unfolded protein response (UPR) (Holcik and Sonenberg, 2005). UPR is an integrated intracellular signal transduction pathway mediated by three ER-resident stress sensors: double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring kinase-1 (IRE1) (Lin et al., 2008;Chakrabarti et al., 2011). Activation of the three proximal sensors is regulated by a member of heat shock protein-70 (HSP-70) family of chaperones, BiP/GRP78. Normally, each of these sensors is bound to GRP78 in the lumen of the ER (Figure 1.2). However, upon accumulation of unfolded and misfolded proteins, BiP dissociates from these sensors. Dissociated PERK homodimerizes and becomes autophosphorylated. Phospho-PERK (P-PERK) then phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2 α), resulting in a global reduction in translation and a switch to translation of proteins required for cell survival under ER

stress such as activated transcription factor 4 (ATF4) (Marciniak and Ron, 2006;Shore et al., 2011). ATF4 drives the expression of pro-survival genes encoding proteins involved in amino acid biosynthesis and transport as well as antioxidants (Harding et al., 2003;Holcik and Sonenberg, 2005;Chakrabarti et al., 2011). Upon dissociation from BiP, pATF6 (90 kDa) translocates to the Golgi compartment where it is processed to its active form through cleavage by site-1 and site-2 proteases (S1P and S2P). The 50-kDa cytosolic fragment migrates to the nucleus and activates transcription of target genes encoding proteins required for alleviation of ER stress such as ER resident molecular chaperones and the ER associated degradation (ERAD) machinery (Ye et al., 2000;Nadanaka et al., 2004;Shen and Prywes, 2005;Zhang and Kaufman, 2008). Dissociation of BiP from IRE1 leads to homodimerization and trans-autophosphorylation. Activated IRE1 functions as endoribonuclease to remove a small intron from X-box binding protein1 (XBP1) mRNA. Spliced XBP1 mRNA encodes a transcription factor that increases the expression of genes encoding enzymes that facilitate protein folding or degradation. ER homeostasis is re-established through the activation of UPR. However, when ER stress is too severe or prolonged, the pro-survival function of the UPR turns into a toxic signal, which is predominantly executed by mitochondrial apoptosis (Zhang and Kaufman, 2008;Chakrabarti et al., 2011).

Figure 1.2: ER stress signaling pathway

ER stress signaling is principally mediated by ER-resident stress sensors: PERK, ATF6 and IRE1, which are maintained in an inactive state by binding to the ER chaperone BiP. Accumulation of unfolded proteins in the ER leads to dissociation of BiP from these sensors and initiation of ER stress signal transduction. Dissociated PERK homodimerizes and becomes autophosphorylated. Phospho PERK phosphorylates eIF2 α leading to a generalized inhibition of translation initiation with preferential translation of the transcription factor, ATF4. ATF6 translocates to the Golgi apparatus following activation where it is cleaved into an active transcription factor. Activated IRE1 functions as endoribonuclease which cleaves XBP1 mRNA to spliced XBP1(S-XPB1) mRNA which encodes for a transcription factor. Under mild ER stress, the ATF4, ATF6, and XBP1 transcription factors increase the expression of ER resident chaperones and ERAD machinery proteins that are required to alleviate ER stress. During extensive ER stress, CHOP10 expression is increased resulting in apoptosis.



1.5.1. ER stress-induced apoptosis

The predominant trigger of ER stress-mediated apoptosis is C/EBP homologous protein (CHOP10), also known as growth arrest and DNA damage-inducible gene 153 (GADD153). CHOP10 transcription is regulated at least by four cis-acting elements, AARE1, AARE2, ERSE1, and ERSE2. The three transcription factors that are activated in response to ER stress (ATF4, P50-ATF6, and S-XBP1) are known to transactivate CHOP10 (Figure 1.2). ATF4 binds to and activates AARE1 and AARE2. Both p50-ATF6 and XBP1 bind to and activate ERSE1 and ERSE2 (Oyadomari and Mori, 2004). CHOP10 is a transcription factor that regulates the expression of pro- and anti-apoptotic genes. Under conditions of prolonged or high level CHOP10 expression, GADD34 is expressed and interacts with protein phosphatase 1 (PP1) to dephosphorylate P-eIF2 α . This leads to a restoration in global protein translation and an increase in the expression of pro-apoptotic genes including death receptor 5 (DR5) (Yagi et al., 2003; Marciniak et al., 2004; Lim et al., 2009). In addition CHOP10 represses the transactivation of anti-apoptotic genes including Bcl-2 (McCullough et al., 2001) thus creating conditions, which favor apoptosis.

1.5.2. ER stress as an anticancer strategy

It is well established that tumor cells are under stress due primarily to the increased levels of hypoxia and decreased levels of glucose (Luo et al., 2009). Consequently, limited energy is available while the rate of protein processing for cell proliferation is high. As an adaptive response, the UPR is activated to counteract the ongoing stress by accommodating the increased protein folding load (Luo et al., 2009; Hsiao et al., 2009). It is important to note that ER stress is generally inactive in most normal cells (except those with a high demand on the ER such as insulin secreting pancreatic cells and antibody producing B-cells) (Healy et al., 2009). There are

two approaches to target ER stress. One approach is to inhibit the ER stress so tumor cells can no longer accommodate the increased protein folding load, thereby leading to cell death. However, the risk exists that the anticancer drug in question might actually block ER stress-mediated apoptosis. The second approach is to expose the tumor cells to overwhelming ER stress, which will drive the cells beyond their capacity to regulate the stress and activate cell death pathway (Healy et al., 2009;Verfaillie et al., 2010;Johnson et al., 2011). This difference in the endogenous ER stress levels allows one to induce cytotoxic ER stress in tumor but not normal cells using ER stress inducing agents. As such, therapeutic benefit can result from targeting this signaling pathway.

Since J-series PG-induced apoptosis can be mediated by activation of the UPR (Takahashi et al., 1998;Saito et al., 2003;Weber et al., 2004;Chambers et al., 2007) and anandamide is metabolized by COX-2 in tumorigenic keratinocytes to J-series PGs, it is plausible that AEA produces overwhelming stress driving the tumor but not normal cells toward apoptosis.

1.6. Endogenous cannabinoids (endocannabinoids)

Endocannabinoids are neuromodulatory lipids that regulate physiologic and pathologic processes including appetite, mood, learning/memory, pain sensation and inflammation by activating the endogenous cannabinoid (endocannabinoid) system (ECS). ECS includes (i) endocannabinoids, which mimic many of the effects of phytocannabinoids such as Δ^9 tetrahydrocannabinol (Δ^9 THC), the active ingredient of *Cannabis sativa* (marijuana), (ii) molecular transporters, (iii) cannabinoid receptors; cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R) and (iv) the enzymes involved in cannabinoid synthesis and degradation (Bisogno et al., 2005).

1.6.1. Anandamide

Anandamide (aka arachidonyl ethanolamide, AEA) is a prototype endocannabinoid. AEA binds CB1 and CB2 receptors and then is transported into cells via the anandamide membrane transporter (AMT). The action of AEA is then terminated by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). Cannabinoid receptors seem to play an important role in the regulation of the anticancer activity of AEA. Several studies showed that AEA inhibits the proliferation of breast and prostate cancers in a CB1 receptor-dependent manner (De et al., 1998; Melck et al., 1999; Melck et al., 2000; Nithipatikom et al., 2011). However, studies conducted in our lab and others revealed that AEA-induced cell death can occur by receptor-independent pathways (Patsos et al., 2010; Kuc et al., 2012).

1.6.1.1. Anandamide metabolism

Fatty acid amide hydrolase (FAAH) hydrolyzes AEA to arachidonic acid (AA) plus ethanolamine (Deutsch and Chin, 1993). Consequently, FAAH terminates the biological activity of AEA (Bifulco et al., 2004; Siegmund et al., 2006; Kuc et al., 2012). Because AEA has an unmodified arachidonate backbone (Figure 1.3), AEA is also susceptible to oxidative metabolism by oxygenases that metabolize AA such as lipoxygenase (LOX), cytochrome P450 and cyclooxygenases (COX). For example, 12- and 15-LOX metabolize AEA to 12(S)-hydroxy-AEA [12(S) HAEA] and 15-HAEA (Edgmond et al., 1998). CYP2J2, CYP2B6 and CYP2D6 were reported to metabolize AEA to epoxyeicosatrienoic acids ethanolamide (EET-EA) and hydroxyeicosatetraenoic acid ethanolamide (HETE-EA) (Snider et al., 2009; Sridar et al., 2011; McDougle et al., 2014). In addition, COX-2 metabolizes AEA to prostaglandin-ethanolamides (PG-EAs) (Figure 1.4), including PGH₂-EA, which is then metabolized to PG-EAs of E, F_{2α}, and D by selective prostaglandin synthases (Yu et al., 1997; Kozak et al., 2002a).

Our lab reported, for the first time, that ethanolamide conjugated J-series PGs are also produced from anandamide (Figure 1.4) (Soliman et al., 2014). Studies conducted by Kozak et al., 2001 reported that the PG-EAs produced from AEA are more metabolically stable than free acid PGs and also suggested that these molecules might have unique activity (Kozak et al., 2001). However, the biological activity of many of the metabolic products of AEA is still unknown.

Figure 1.3: Chemical structure of Arachidonic acid and Anandamide

The chemical structure of AA and AEA is obtained from reported studies (Wiley et al., 2006) .

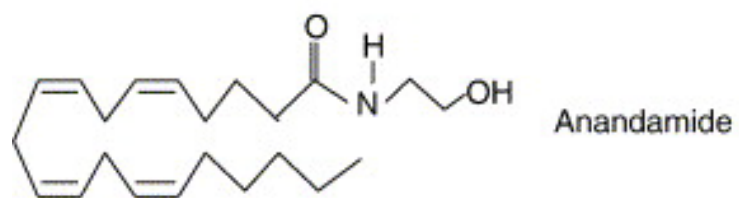
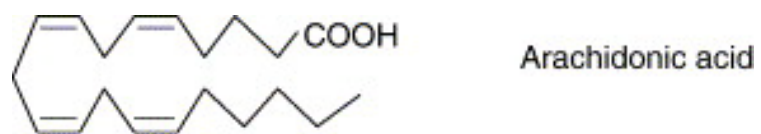
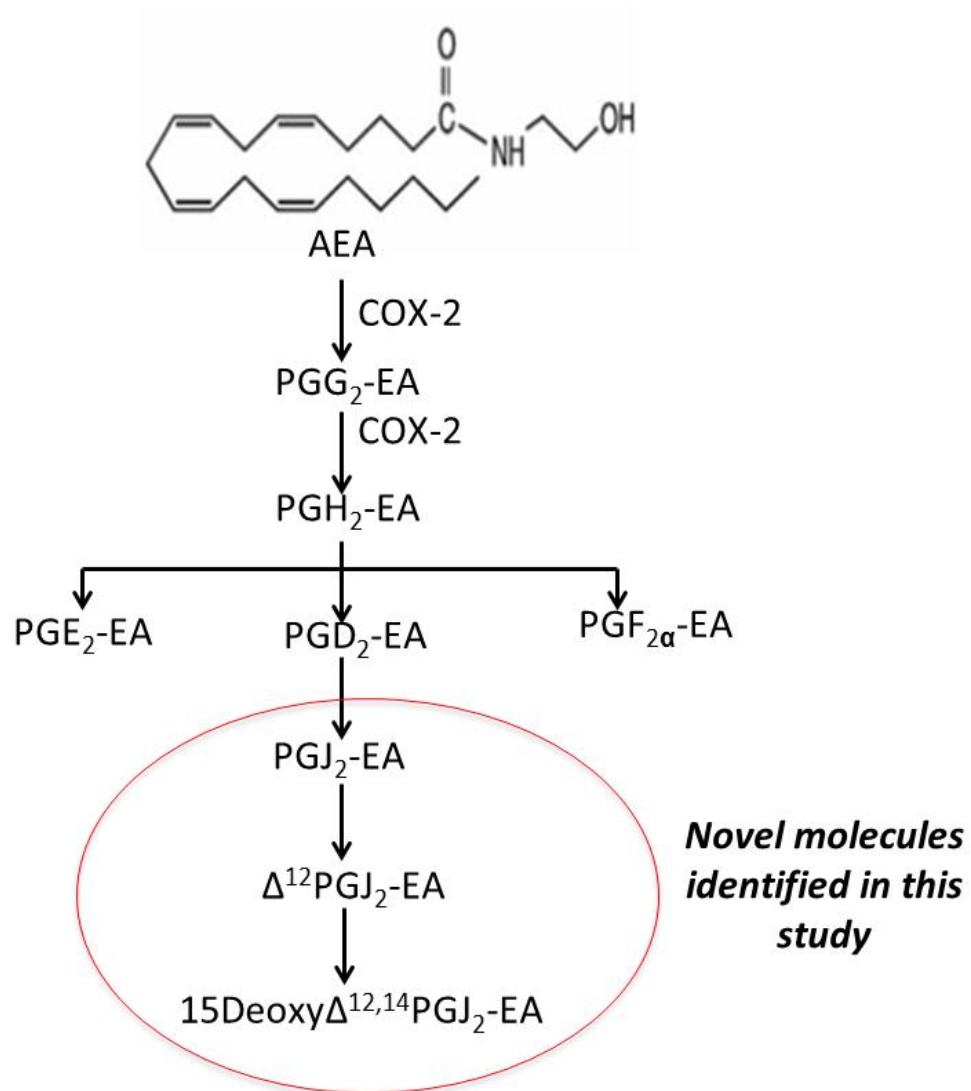


Figure 1.4: Metabolism of Anandamide by COX-2

COX-2 metabolizes AEA to PGH₂-ethanolamide (PGH₂-EA), which is then metabolized to PGD₂-EA, PGE₂-EA, and PGF_{2 α} -EA by selective prostaglandin synthases. PGD₂-EA is then dehydrated to PGJ₂-EA, Δ^{12} PGJ₂, and 15-deoxy $\Delta^{12,14}$ PGJ₂-EA.



1.7. Specific Aims

The antiproliferative effect of anandamide (aka arachidonyl ethanolamide, AEA) on tumorigenic keratinocytes (JWF2) is dependent upon the metabolism of AEA by cyclooxygenase-2 (COX-2) to PGJ-EAs (Van Dross, 2009;Soliman et al., 2014). In addition, oxidative stress mediates AEA-induced apoptosis (Kuc et al., 2012). However, a detailed identification of the signaling molecules and pathways involved in AEA-induced apoptosis in tumorigenic keratinocytes was needed. To begin to understand how AEA causes apoptosis, two important signal transduction pathways, ER stress and oxidative stress, were examined. It is well established that ER stress and oxidative stress are key regulators of cell death in a variety of tumor cell types. Several cannabinoids and AA-derived, J-series prostaglandins induce oxidative stress, ER stress and apoptosis (Chambers et al., 2007;Su et al., 2008). Therefore, the present study hypothesized that the J-series metabolic products of AEA initiated ER stress-induced apoptosis through the induction of oxidative stress. Furthermore, since our previous data indicated that AEA-induced cell death did not require cannabinoid receptor activation we hypothesized that oxidative stress, ER stress and apoptosis would also occur independent of these receptors. To examine these hypotheses, the following Specific Aims were developed.

Specific Aim 1

Studies in Specific Aim 1 determined the role of COX-2, J-series prostaglandins, and ER stress in AEA-induced apoptosis.

Rationale:

AEA causes cell death in a variety of tumor cell lines. AEA-induced cell death was controlled by COX-2 in colon and skin cancer cells (Patsos et al., 2010;Kuc et al., 2012). COX-

2 metabolized AEA to PGE₂-EA, PGF_{2 α} -EA and PGD₂-EA. J-series prostaglandins were also found to be metabolic products of COX-2 (Van Dross, 2009). In addition, J-series prostaglandin-induced cell death was mediated by ER stress. Hence, the role of COX-2, J-series prostaglandins and ER stress in AEA-induced apoptosis was examined by addressing the following questions:

- 1- Does AEA activate the apoptotic ER stress response in tumorigenic keratinocytes?
- 2- Is apoptotic ER stress signaling required for AEA-induced apoptosis in tumorigenic keratinocytes?
- 3- Is COX required for AEA-induced ER stress-apoptosis?
- 4- What is the identity of the J-series prostaglandins that are derived from AEA?
- 5- Is 15d-PGJ₂-EA required for AEA-induced ER stress-apoptosis?

Specific Aim 2

Studies in Specific Aim 2 determined if oxidative stress was required for AEA-induced ER stress-apoptosis. In addition, the role of CB1R and CB2R in AEA-mediated oxidative stress, ER stress and apoptosis was examined.

Rationale:

Cancer cells produce relatively high levels of ROS, which can activate pro-survival signal transduction pathways. Excessive ROS production may cause irreversible oxidative damage that leads to apoptosis in cancer cells (Mates et al., 2012; Glasauer and Chandel, 2014). Oxidative stress was required for AEA-induced apoptosis in tumorigenic keratinocytes (Kuc et al., 2012).

Also, AA-derived J-series PGs caused cell death by generating excessive oxidative stress. Oxidative stress induced by the J-series prostaglandins is mediated by a decrease in GSH and an increase in ROS (Kondo et al., 2001;Kondo et al., 2002;Uchida and Shibata, 2008). In addition, oxidative stress is an important initiator of endoplasmic reticulum (ER) stress (Cao and Kaufman, 2014). The ER is an organelle in which oxidative protein folding occurs, therefore alterations in redox status affect ER homeostasis, protein folding, and ER stress-apoptosis (Malhotra and Kaufman, 2007;Tabas and Ron, 2011). The structure of J-series PG ethanolamides is similar to AA-derived J-series prostaglandins therefore these molecules may exhibit similar properties. In addition, AEA-mediated cell death occurs through cannabinoid receptor-dependent and -independent pathways. Therefore, we evaluated the role of oxidative stress and the cannabinoid receptors in AEA-induced ER stress-apoptosis by examining the following questions:

- 1- Is oxidative stress required for AEA-induced ER stress-apoptosis?
- 2- What is the mechanism by which AEA induces oxidative stress?
- 3- Is the activation of endocannabinoid receptors CB1 or CB2 required for AEA-induced apoptosis?
- 4- Is the activation of endocannabinoid receptors CB1 or CB2 required for AEA-induced oxidative and ER stress?

CHAPTER TWO: MATERIALS AND METHODS

2.1. Antibodies and reagents

AEA, 15-deoxy- $\Delta^{12,14}$ PGJ₂, PGD₂-EA, AM251, AM630 and PGD₂ ELISA kits were purchased from Cayman Chemical Company (Ann Arbor, MI). 15-deoxy- $\Delta^{12,14}$ PGJ₂ ELISA kits were purchased from Assay Designs (Ann Arbor, MI). Anti-COX-2-TRITC, goat anti-lamin B1 and anti-COX-2 and rabbit anti-CHOP10 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-FAAH, anti-P-PERK, anti-t-PERK, anti-P-eIF2 α , anti-t-eIF2 α , and anti-t-IRE1 were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-full length (FL) caspase-3 was from Invitrogen (Carlsbad, CA) and anti-GAPDH was from Trevigen (Gaithersburg, MD). Rabbit anti-P-IRE1 was purchased from Abcam (Cambridge, MA). Anti-rabbit Alexafluor 488 and anti-goat Alexafluor 555 were from Invitrogen (Grand Island, NY) while anti-goat Alexafluor 647 was from Jackson Immunoresearch (West Grove, PA). Anti-goat IRDye 800CW, anti-rabbit 800CW, anti-mouse 800CW, and anti-mouse 680RD were from Li-cor Biosciences (Lincoln, Nebraska). Rabbit anti-H-PGDS and anti-L-PGDS were purchased from Cayman Chemical Company (Ann Arbor, MI). N-Acetyl Cysteine, and Trolox were purchased from Sigma-Aldrich (St. Louis, MO). Anti-ATF6 (TO 13/14) was a gift from Dr. Allen Volchuk, Toronto General Research Institute.

2.2. Cell culture

The murine squamous carcinoma cell line JWF2 (a kind gift from Dr. Susan Fischer; MD Anderson Cancer Center, Smithville, TX) was cultured in Eagle's minimal essential medium (US Biological) containing 5% heat-inactivated fetal bovine serum, penicillin (100 mg/ml), streptomycin (100 mg/ml), nonessential amino acids, and glutamine. Human keratinocytes, HaCaT (Cell Line Service, Germany) and human colorectal cancer cells, HCA7

(Sigma-Aldrich, MO) were cultured in Dulbecco's minimal essential media (Invitrogen) containing 10% heat-inactivated FBS, penicillin (100 mg/mL), streptomycin (100 mg/mL), and sodium pyruvate (1mM). HT29 cells (a kind gift from Dr. Jean-Luc Scemama, Department of Biology, East Carolina University) were cultured in McCoy's 5A media (Sigma-Aldrich, MO) containing 10% heat-inactivated fetal bovine serum, penicillin (100 mg/ml), streptomycin (100 mg/ml).

2.3. Liquid Chromatography-Mass Spectrometry (LC-MS)

Prostaglandins were extracted from acidified cell culture medium using solid phase extraction (SPE). After the sample was applied to the equilibrated cartridge, the packing bed was washed with ultrapure deionized water, followed by 15% acetonitrile and 100% hexane. Prostaglandins were eluted in 100% acetonitrile. Extracted prostaglandins were evaporated to dryness under a stream of nitrogen gas and reconstituted in 10 μ L of acetonitrile.

LC-MS was performed on a Waters Acquity Ultra-High Performance Liquid Chromatograph paired with a Micromass Quadrupole/Time-of-Flight Mass Spectrometer equipped with an Electrospray Ionization (ESI) source (Milford, MA). The LC was equipped with an Acquity C₈ column (2.1 mm i.d., 100 mm length, particle diameter 1.7 μ M). Mobile phase composition was solvent A (90/10 v/v water/acetonitrile) and solvent B (100% acetonitrile). The gradient began at 65% A/35% B; it was lowered to 50% at 5%/minute, then to 35% A at 7.5%/minute at a constant flow rate of 0.4 mL/minute. Eluent was introduced into the ESI source, which is heated to 275°C, using a cone gas (nitrogen) flow rate of 10 L/hr. Inside the ESI source, heated nitrogen gas (120°C, flow rate 500 L/hr.) aided in desolvation. The ESI source was operated in negative ion mode. For LC-MS/MS experiments, parent ions were mass-

selected in the quadrupole and were subjected to collision-induced dissociation (CID) in the hexapole collision cell. Collision energy was 25 eV and argon was the collision gas.

2.4. Transfections and luciferase assays

HaCaT cells were cultured in chamber slides and transfected with human COX-2 cDNA in pcDNA6.1 (pcDNA6.1-h-COX-2) or pcDNA6.1 with no insert (empty vector) using Lipofectamine LTX and Plus Reagent (Invitrogen). For luciferase assays, HaCaT cells were co-transfected with the ER stress responsive element (ERSE) luciferase reporter and the Renilla luciferase expression plasmid (Qiagen Sciences). The total DNA concentration transfected into HaCaT cells was equivalent. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

2.5. XBP splicing assay

Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out using standard procedures (Sambrook et al., 1989). JWF2 cells were treated as indicated, RNA isolated with Trizol Reagent and 1 µg of total RNA from each sample treated with DNase I followed by first strand synthesis with MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). XBP1 forward 5'-GAACCAGGAGTTAAGAACACG-3' and reverse 5'-AGGCAACAGTGTCTCAGAGTCC-3' PCR primers (Samali et al., 2010) amplify both unspliced (205bp) and spliced (179bp) XBP1. GAPDH forward and reverse primers are 5'-GTCTACTGGTGTCTTCACCA-3' and 5'-GTGGCAGTGATGGCATGGAC-3' (Van Dross et al., 2007), respectively. PCR products were resolved on a 3% MetaPhor agarose gel (Cambrex BioScience).

2.6. Western blot analysis

Western blot analysis was conducted as described previously (Van Dross, 2009). JWF2 cells were plated in 100 mm tissue culture plates for 48 hours and treated as indicated in serum-free culture medium. Cells were washed twice with phosphate buffer saline (PBS) then 100 μ l triton lysis buffer (TLB) (1% Triton, 25mM Tris pH 7.4, 150mM NaCl + protease phosphatase inhibitors mix) was added. Whole cell lysate was centrifuged at 14,000 rpm (20,000 x g) for 20 min and the supernatant was used for the determination of protein concentration using BCA reagents (Thermo Fisher Scientific Inc., IL, USA). Equal concentrations of each sample were loaded on to SDS-PAGE gels and protein bands transferred to nitrocellulose membranes (BioRad, Hercules, CA) in the semi-dry transfer cell TRANS-BLOTSD (Bio-Rad Laboratories, Hercules, CA) using transfer buffer (containing 230 mM glycine, 25mM Tris, 0.7 mM SDS, 20% methanol) at 26 volts for one hour. Nonspecific binding sites on the membranes were blocked at room temperature using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for two hours. Membranes were then incubated with the indicated primary antibodies overnight and the appropriate secondary antibody for one hour. Protein bands were visualized using the Li-cor system and digitized images quantified using Image-J software.

2.7. Immunofluorescence

Cells were treated as indicated, fixed with methanol, incubated with permeabilization buffer (0.1% Triton X-100 in 1xPBS) for 10 minutes, and then blocked with blocking buffer (1X PBS + 3% FBS + 0.5% Tween20) for 1 hour. The blocked cells were then incubated with the indicated primary antibodies and the appropriate immunofluorescence-tagged secondary antibodies. Images were acquired and analyzed by confocal laser microscopy (ZEISS LSM 510

confocal microscope system). Fluorescence intensity was quantified using Zen Lite 2011 software.

2.8. D- and J-series prostaglandin ELISA assays

The culture medium from agent-treated JWF2 cells was collected and D- and J-series prostaglandins measured with ELISA kits as described by the manufacturers.

2.9. MTS cell viability assay

JWF2 cells were plated in 96-well plates and cultured for 48 h. Media containing the appropriate concentration of different agents were added to the cells for the indicated time. 20 μ l MTS reagent (Promega, Madison, WI) was then added to each well as directed by the manufacturer and absorbance measured at 495 nm.

2.10. Oxidative stress measurement

The oxidative stress in JWF2 cells was measured by chloromethyl-2',7' dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) staining. Cells were treated with 5 μ M CM-H₂DCFDA (Molecular Probes, Invitrogen) in phenol red-free, serum-free medium for 30 min and then treated with the appropriate drug for the indicated time. Cells were then trypsinized and suspended in PBS. DCF fluorescence was measured using an Accuri C6 flow cytometer (BD Accuri Cytometers, Ann Arbor, MI, USA) at an excitation wavelength of 488 and a 533 \pm 30-nm emission filter.

2.11. Flow cytometry analysis of P-eIF2 α

Treated cells were trypsinized, resuspended in PBS, centrifuged, and the cell pellet reconstituted in 4% formaldehyde in PBS. The cells were then fixed for 10 min at 37⁰C and samples permeabilized with methanol (the final concentration is 90% methanol). Cells were then

washed using incubation buffer, counted, aliquoted (1×10^6 cells/sample), and blocked in incubation buffer. Next, JWF2 cells were exposed to P-eIF2 α antibody followed by Alexafluor 555 secondary antibody (Life technologies, Invitrogen). The mean fluorescence of 10000 life events was detected with Accuri C6 flow cytometer (BD Accuri Cytometers, Ann Arbor, MI, USA) at an excitation wavelength of 488 and a 585 \pm 40-nm emission filter.

2.12. Total glutathione measurement

JWF2 cells were plated in 100 mm plates and cultured for 48 h. Media containing the appropriate treatments were added to the cells for the indicated time. Cells were washed with PBS and kept at -80 $^{\circ}$ C until the time of performing the assay. Cells were lysed in TEE buffer containing: 10 mM Tris base, 1 mM EDTA and 1 mM EGTA, with 0.5% Tween-20 and 1% triton X-100. Total glutathione measurements were performed using Tietze method (Tietze, 1969) with some modifications. Cell lysate was subjected to centrifugation at 10,000 rpm for 10 minutes at 4 $^{\circ}$ C. The assay was performed in a 96 well plate by adding 25 μ l of sample or oxidized glutathione standard (GSSG) (5 μ M, 2.5 μ M, 1.75 μ M, 0.875 μ M and 0.437 μ M) followed by 50 μ l of chromagen (freshly prepared 1:1 solution of 1mM 2,2'-Dithiobis(5-nitropyridine) (DTNP) and Glutathione reductase). DTNP (Sigma-Aldrich, St. Louis, MO) was prepared in 100mM Tris base buffer, pH 8.35) and Baker's yeast Glutathione reductase (Sigma-Aldrich, St. Louis, MO) was diluted 100 times in TEE buffer containing: 10 mM Tris base, 1 mM EDTA and 1 mM EGTA, with 0.5% Tween-20. The mixture was incubated at room temperature for 15 minutes and then 25 μ l 10 mM NADPH (Sigma-Aldrich, St. Louis, MO) was added. The NADPH solution was prepared in 100mM Tris base, pH 8.35, aliquots were stored at -80 $^{\circ}$ C. Immediately after the addition of NADPH, the absorbance was measured kinetically (every 1 minute for 5 minutes) at 405 using the BioTek, Synergy HT plate reader.

2.13. Cyclic AMP measurement:

JWF2 cells were plated in 96-well plates and cultured for 48 h. Cells were pretreated with appropriate concentration of cannabinoid receptor antagonists for 1 hour and then treated with AEA for 15 minutes. cAMP was measured using cAMP-Glo Max Assay kit (Promega, Madison, WI) according to manufacturer's protocol. Luminescence was determined with a luminometer and used as a measure of cAMP produced. For each sample: the change in relative luminescence units (ΔRLU) = RLU (untreated sample) – RLU (treated sample) was calculated. Using this ΔRLU value and the linear equation generated from the standard curve, the cAMP concentration was calculated.

2.14. Statistical analysis

All data are representative of three independent experiments. Data are presented as mean \pm Standard error of mean (SEM). Student's T-test and Analysis Of Variance (ANOVA) followed by Tukey's post-hoc analysis were carried out using GraphPad Prism and Microsoft Excel.

**CHAPTER THREE: ARACHIDONOYL-ETHANOLAMIDE ACTIVATES
ENDOPLASMIC RETICULUM STRESS-APOPTOSIS IN TUMORIGENIC
KERATINOCYTES: ROLE OF CYCLOOXYGENASE-2 AND NOVEL J-SERIES
PROSTAMIDES**

3.1. ABSTRACT

Non-melanoma skin cancer and other epithelial tumors overexpress cyclooxygenase-2 (COX-2), differentiating them from normal cells. COX-2 metabolizes arachidonic acid to prostaglandins including the J-series prostaglandins, which induce apoptosis by mechanisms including endoplasmic reticulum (ER) stress. Arachidonoyl-ethanolamide (AEA) is a cannabinoid that causes apoptosis in diverse tumor types. Previous studies from our group demonstrated that AEA was metabolized by COX-2 to J-series prostaglandins. Thus, the current study examines the role of COX-2, J-series prostaglandins, and ER stress in AEA-induced apoptosis. In tumorigenic keratinocytes that overexpress COX-2, AEA activated the PKR-like ER kinase (PERK), inositol requiring kinase-1 (IRE1), and activating transcription factor-6 (ATF6) ER stress pathways and the ER stress apoptosis-associated proteins, C/EBP homologous protein-10 (CHOP10), caspase-12, and caspase-3. Using an ER stress inhibitor, it was determined that ER stress was required for AEA-induced apoptosis. To evaluate the role of COX-2 in ER stress-apoptosis, HaCaT keratinocytes with low endogenous COX-2 expression were transfected with COX-2 cDNA or an empty vector and AEA-induced ER stress-apoptosis occurred only in the presence of COX-2. Moreover, LC-MS analysis showed that the novel prostaglandins, 15-deoxy $\Delta^{12,14}$ PGJ₂-EA and Δ^{12} PGJ₂/PGJ₂-EA, were synthesized from AEA. These findings suggest that AEA will be selectively toxic in tumor cells that overexpress COX-2 due to the metabolism of AEA by COX-2 to J-series prostaglandin-ethanolamides (prostamides).

Hence, AEA may be an ideal topical agent for the elimination of malignancies that overexpress COX-2.

3.2. INTRODUCTION

Non-melanoma skin cancer (NMSC) is the most common cancer in the United States. In 2004, NMSCs were associated with estimated direct costs of \$1.4 billion and indirect costs of \$76.8 million. These costs are expected to rise dramatically because NMSC incidence is increasing due to excessive sun and artificial UV-light exposure. Therefore, novel treatments and improved prevention strategies are needed to address this issue.

A number of intriguing studies show that chemotherapeutic benefits may result from targeting the endoplasmic reticulum (ER) stress signaling pathway (reviewed in (Healy et al., 2009)). ER stress is caused by elevated protein folding loads, intracellular calcium imbalance and other cellular stresses which cause unfolded or misfolded protein accumulation (Boelens et al., 2007). In response to this, an intracellular signal transduction pathway, the unfolded protein response (UPR), alleviates ER stress to promote cell survival, or initiates cell death if the ER stress machinery is critically overloaded. In tumor cells, moderate levels of ER stress are present due to increased proliferation rates, excessive protein processing, and limited energy (Luo et al., 2009;Hsiao et al., 2009). Therefore, a therapeutic approach is to expose the tumor cells to ER stress inducers to overload the ER machinery thereby inducing cell death (Healy et al., 2009;Verfaillie et al., 2010). In contrast, ER stress-mediated cell death is difficult to achieve in normal cells which typically contain low endogenous levels of ER stress (Healy et al., 2009).

The survival and cytotoxic ER stress pathways are regulated by three ER-resident stress sensors: double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring kinase-1 (IRE1). PERK is activated by autophosphorylation and phospho-PERK (P-PERK) phosphorylates the alpha subunit of eukaryotic initiation factor 2 α (eIF2 α) to attenuate global mRNA translation and

decrease the ER protein folding load (Marciniak and Ron, 2006;Shore et al., 2011). Activated ATF6 (90 kDa) translocates to the Golgi compartment where it is cleaved to a 50-kDa fragment that accumulates in the nucleus to increase the expression of genes encoding protein chaperones which alleviate ER stress (Shen and Prywes, 2005;Zhang and Kaufman, 2008). Also, IRE1 becomes phosphorylated and functions as an endoribonuclease that removes a small intron from X-box binding protein1 (XBP1) mRNA. XBP1 encodes a transcription factor that increases the expression of chaperones and enzymes involved in protein degradation thereby resolving ER stress (Zhang and Kaufman, 2008;Chakrabarti et al., 2011). Alternatively, in the presence of prolonged or overwhelming ER stress, these activated signaling pathways induce apoptosis by causing maximal induction of C/EPB homologous protein10 (CHOP10) transcription primarily via the ER stress responsive element (ERSE) and the amino acid responsive element (AARE) in the CHOP10 promoter (Oyadomari and Mori, 2004). In addition, ER stress mediated apoptosis is triggered by ER resident caspase-12 (mouse) or caspase-4 (human), which do not activate apoptosis in the absence of ER stress (Nakagawa et al., 2000;Szegezdi et al., 2003). Various agents with antineoplastic activity overload these ER stress pathways and initiate cell death, including proteasome inhibitors (Maria et al., 2013), NSAIDS (Du et al., 2011) and cannabinoids (Carracedo et al., 2006;Mecha et al., 2012).

Cannabinoids are bioactive signaling lipids that bind to cannabinoid receptors (CBR) to modulate processes such as pain and inflammation. Cannabinoids also regulate tumor survival by increasing cell death, inducing growth arrest, and inhibiting angiogenesis and metastasis (Grimaldi et al., 2006;Ramer and Hinz, 2008;Miyato et al., 2009;Patsos et al., 2010). Arachidonoyl-ethanolamide (AEA) is an endogenously synthesized cannabinoid that causes tumor cell death via receptor-dependent or -independent mechanisms (Alpini and DeMorrow,

2009;Brown et al., 2010;Van Dross et al., 2012). Several cellular proteins regulate receptor-independent AEA death including cyclooxygenase-2 (COX-2) (Van Dross, 2009;Kuc et al., 2012).

COX-2 is an enzyme that is overexpressed in NMSCs and their precursor lesions and also promotes carcinogenesis (Buckman et al., 1998). Clinical and animal tumor studies revealed that COX-2 inhibition decreases tumor development (Wilgus et al., 2003;Elmets et al., 2010). COX-2 is the rate-limiting enzyme in prostaglandin (PG) synthesis from arachidonic acid (AA). COX-2 oxygenates AA to PGH₂ which is then metabolized to PGE₂, PGF_{2α}, and PGD₂, by prostaglandin synthase-E, -F, and -D, respectively. Subsequently, PGD₂ is dehydrated to J-series prostaglandins. J-series prostaglandins are well-known inducers of cell death (Clay et al., 1999;Wang and Mak, 2011) and these cytotoxic lipids can cause apoptosis via ER stress (Takahashi et al., 1998;Chambers et al., 2007). Interestingly, COX-2 metabolizes the cannabinoid AEA to ethanolamide (EA)-conjugated PGH₂ which is then converted to PGE₂-EA, PGF_{2α}-EA, and PGD₂-EA by prostaglandin synthases (Yu et al., 1997;Kozak et al., 2002b). We previously determined that AEA was metabolized by COX-2 to J-series prostaglandins (25). However, it has not been determined if AEA-mediated apoptosis is caused by COX-2-dependent metabolism of AEA to J-series prostaglandins or by the induction of ER stress. Furthermore, the chemical identity of the J-series prostaglandins that are derived from AEA is also unknown. Hence, the current study examined the role of COX-2, J-series prostaglandins and ER stress in AEA-mediated apoptosis. The results showed that AEA activated ER stress and apoptosis in a COX-2-dependent manner. Moreover, we identified J-series prostaglandin-ethanolamides (prostamides) as a novel subclass of prostaglandins that likely play a role in AEA-mediated apoptosis. Since COX-2 is overexpressed in tumor but not normal keratinocytes, our

findings suggest that AEA will be selectively converted to cytotoxic J-series prostamides in tumor cells thereby causing minimal toxicity in non-tumor cells.

3.3. RESULTS

3.3.1. AEA was selectively toxic to tumorigenic keratinocytes

To confirm our previous finding that AEA induces cell death selectively in NMSC cells which overexpress COX-2, tumorigenic (JWF2) and non-tumorigenic (HaCaT) keratinocytes were treated with different concentrations of AEA or vehicle for 24 hours and cell viability was measured using MTS assays. A significant reduction in cell viability was observed in JWF2 cells but not in HaCaT cells treated with 20 μ M AEA. HaCaT cell viability was 35.7-fold higher than that of JWF2 cells in the presence of 20 μ M AEA (Figure 3.1A). To assess whether the differential cytotoxicity of AEA in JWF2 and HaCaT cells might be associated with COX-2 or FAAH, the expression of these enzymes was examined. AEA is catabolized by FAAH to AA plus ethanolamine. Our previous data suggested that FAAH inhibition prevented AEA degradation thereby increasing the quantity of AEA that is available for metabolism by COX-2 to J-series PGs (Kuc et al., 2012). FAAH expression levels were low in both tumorigenic (JWF2) and non-tumorigenic (HaCaT) keratinocytes (Figure 3.1B). On the other hand, COX-2 was overexpressed in JWF2 but not HaCaT cells. This suggests that JWF2 cells were more susceptible to cell death due to the metabolism of AEA by COX-2 to J-series PGs.

3.3.2. AEA induced cell death in tumor cell lines with high COX-2 and low FAAH expression

The cytotoxicity of AEA was then examined in tumorigenic cell lines with variable levels of COX-2 and FAAH expression. Two human colorectal cancer cell lines, HT29 and HCA7 were treated with increasing concentrations of AEA for 24 hours and then cell viability was measured by conducting MTS assay. As shown in Figure 3.2A-B, 20 μ M AEA induced cell death in HCA7 cells (Figure 3.2B), however, HT29 (Figure 3.2A) were resistant to AEA

cytotoxicity. To determine if AEA-induced cytotoxicity correlated with the magnitude of COX-2 and FAAH expression, Western blot analysis was conducted. As shown in Figure 3.2C, COX-2 expression was highest in HCA7 but moderate in HT29. However, the level of FAAH expression was higher in HT29 when compared to HCA7 cells. These data indicate that high COX-2 and low FAAH levels favor AEA cytotoxicity.

3.3.3. AEA induced ER stress in NMSC cells

To evaluate whether AEA induces apoptosis via the ER stress pathway we first examined its ability to activate the ATF6, PERK, and IRE1 ER stress signaling pathways in tumorigenic keratinocytes. JWF2 cells were exposed to 20 μ M AEA or vehicle (ethanol) for 1, 2, or 4 hours and ER stress sensor activation was measured. Immunofluorescence analysis was conducted to investigate ATF6, which translocates from the cytoplasm to the nucleus when activated. In AEA-treated cells, ATF6 was located in the nucleus whereas it was primarily observed in the cytoplasm of vehicle-treated cells (Figure 3.3A). To investigate the activation of PERK, the phosphorylation of PERK and its substrate, eIF2 α were examined by conducting Western blot analysis. PERK and eIF2 α phosphorylation were increased as a result of cell treatment with AEA (Figure 3.3B, left panels). A control experiment with the well-known ER stress inducer, thapsigargin, showed a similar but less intense increase in PERK and eIF2 α phosphorylation (Figure 3.3B, right panels). Activation of IRE1 was detected by examining its autophosphorylation and endoribonuclease activity. IRE1 phosphorylation increased (2 hours) and then decreased (4 hours) in cells exposed to AEA (Figure 3.3C, top panel). By employing RT-PCR analysis we also determined that unspliced (inactive) XBP1 mRNA was reduced in JWF2 cells treated with AEA indicative of P-IRE1 endoribonuclease activity (Figure 3.3C, bottom panel).

Our previous data suggest that the cytotoxic effects of AEA in tumorigenic keratinocytes are dependent on the overexpression of COX-2 and the subsequent synthesis of J-series prostaglandins. Therefore, J-series prostaglandin synthesis was measured to evaluate whether its production occurred in parallel with ER stress. J-series prostaglandin synthesis was significantly increased (Figure 3.3D) in JWF2 keratinocytes, which overexpress COX-2 (Figure 3.1B). J-series prostaglandin synthesis was elevated at 30 minutes (Figure 3.3D) and persisted up to 5 hours (data shown in Figure 3.9A) suggesting that these molecules play an important role in ER stress.

3.3.4. ER stress was required for AEA-induced apoptosis

In the presence of overwhelming ER stress, activated ER stress sensors increase CHOP10 expression which ultimately leads to apoptosis. To determine if AEA increases CHOP10 expression in tumorigenic keratinocytes, JWF2 cells were treated with AEA and Western blot analysis was conducted. AEA caused a substantial increase in CHOP10 expression (Figure 3.4A). To determine if AEA also activated ER stress-associated apoptosis, cells were treated with AEA and the expression of caspase 12 and caspase 3 were measured. AEA caused an increase in both pro-caspase 12 and its active, cleaved product (Figure 3.4B). In addition, caspase 3 cleavage was stimulated in the presence of AEA (Figure 3.4C and 3.4D).

Under conditions of ER stress PERK phosphorylates eIF2 α and phosphorylated eIF2 α (P-eIF2 α) blocks global mRNA translation thus permitting resolution of the ER stress. During insurmountable ER stress, CHOP10 activates PP1/GADD34 phosphatase, which dephosphorylates P-eIF2 α allowing the resumption of mRNA translation. Consequently, the expression of proapoptotic proteins increases and apoptosis occurs (Yagi et al., 2003; Marciniak et al., 2004). Salubrinal is a selective inhibitor of PP1/GADD34, which prevents P-eIF2 α

dephosphorylation and subsequent mRNA translation thereby protecting cells from ER stress (Boyce et al., 2005). To test whether AEA-induced apoptosis is mediated by the ER stress pathway, cells were pretreated with salubrinal. AEA caused an initial increase in the phosphorylation of eIF2 α at 2 hours followed by dephosphorylation at 6 hours. Salubrinal prevented eIF2 α dephosphorylation at the 6 hour time point as anticipated (Figure 3.5A). In addition, salubrinal reduced AEA-induced apoptosis (Figure 3.5B) and cell death (Figure 3.5C) confirming the involvement of ER stress in AEA-induced apoptosis.

3.3.5. AEA was metabolized to J-series prostamides

In this report and our previous study we measured J-series prostaglandin levels by ELISA and found that AEA increased J-series prostaglandin production (Kuc et al., 2012). However ELISA does not provide information about the specific J-series prostaglandins that are synthesized. To determine the identity of the J-series prostaglandins produced in the culture medium of AEA- or ethanol-treated JWF2 keratinocytes, LC-MS analysis was conducted by Allison Danell and Drisheka Thati, chemistry department, ECU. PGJ₂, Δ^{12} PGJ₂, and 15-deoxy $\Delta^{12,14}$ PGJ₂ are commercially available and were used as reference compounds to optimize the procedure for detecting J-series prostaglandins using mass spectrometry. The reference compounds were added to fresh and spent media before and after extraction and yielded similar peak intensities for m/z 333 (PGJ₂, Δ^{12} PGJ₂) and m/z 315 (15-deoxy $\Delta^{12,14}$ PGJ₂) indicating that little to no sample was lost during the enrichment process (data not shown). To identify the J-series prostaglandins synthesized in AEA-treated cells LC-MS and LC-MS/MS was conducted. Novel J-series prostamides (EA, +43 amu) were detected at m/z 358 (15-deoxy $\Delta^{12,14}$ PGJ₂-EA) and m/z 376 (PGJ₂-EA, Δ^{12} PGJ₂-EA). Figure 3.6A and 3.6B show LC-MS/MS spectra of m/z 358 and 376, illustrating the use of MS/MS to confidently identify these m/z values. The insets

in both showed that the ions of interest were detected in the LC-MS spectra of AEA-treated cells (red), but not in vehicle-treated cells (blue). It is important to note that unconjugated J-series prostaglandins (PGJ₂, Δ¹²PGJ₂, and 15-deoxyΔ^{12,14}PGJ₂) were not detected in JWF2 cells treated with AEA (data not shown). This indicated that AA-derived, J-series prostaglandins were not major contributors to towards AEA-induced ER stress and apoptosis and also signified that the J-series prostamides were not subject to cleavage by cellular enzymes.

Because *m/z* 358 was observed in the LC-MS spectra of AEA-treated cells as well as in the LC-MS/MS spectra of *m/z* 376, a control experiment was conducted to approximate the extent of unintended dissociation of *m/z* 376. The parent ion, *m/z* 376, was mass-selected and subjected to collision induced dissociation (CID) at the same collision energy used to transmit ions from the quadrupole to the time-of-flight in LC-MS (5 eV). This low potential focuses the ion beam through the hexapole collision cell to obtain a conventional mass spectrum. Only a low intensity *m/z* 358 peak was observed, indicating *m/z* 376 does not significantly dissociate to *m/z* 358 under the conditions used to obtain the LC-MS spectra (Figure 3.6C). Thus, the majority of the signal at *m/z* 358 arises from the production of 15-deoxyΔ^{12,14}PGJ₂-EA in the AEA-treated cells.

3.3.6. AEA-induced ER stress was COX-2 dependent

Since J-series prostamides are products of the metabolism of AEA by COX-2 our goal was to determine if J-series prostamides mediate AEA-induced ER stress. The rate-limiting enzyme in the production of all prostaglandins and prostamides is COX-2. The role of COX-2 in AEA-induced ER stress and apoptosis however, could not be determined by genetic disruption or pharmacological inhibition of COX-2 because COX-2 inhibition causes tumor cell death (Sheng et al., 1997;Sheng et al., 1998). The use of COX-2 inhibitors is further complicated by the fact

that these agents induce ER stress (Du et al., 2011). Therefore, as an alternative approach the significance of COX-2 in AEA-induced apoptosis was determined by overexpressing COX-2 in keratinocytes with low endogenous levels of COX-2. In AEA-treated cells, a substantial increase in eIF2 α phosphorylation was observed in COX-2 compared with empty vector transfected cells (Figure 3.7A, right panels and Figure 3.7B). In cells treated with AEA, ATF6 was located in the nucleus of COX-2 transfected cells whereas ATF6 was primarily observed in the cytoplasm of cells transfected with the empty vector (Figure 3.7C, right panels and Figure 3.7D). It is interesting to note that in experiments where AEA-treated cells were transfected with COX-2, eIF2 α phosphorylation and ATF6 activation were increased in COX-2 containing cells (Figure 3.7B and 3.7D, inset) as well as in the neighboring cells. This result provides support for the idea that ER stress is caused by J-series prostaglandins, which are known to be secreted from cells and act locally in a paracrine and autocrine manner.

3.3.7. ER stress-mediated apoptosis in AEA-treated cells required COX-2

High magnitude CHOP expression is necessary for the initiation of apoptosis (Okada et al., 2002; Su and Kilberg, 2008). Since CHOP10 expression is regulated by responsive elements including the ERSE, we investigated whether ERSE promoter activity required COX-2. AEA significantly increased ERSE promoter activity in HaCaTs with enforced COX-2 expression compared to empty vector-transfected cells (Figure 3.8A). Consistent with these results, AEA increased CHOP10 protein expression in COX-2-transfected relative to empty vector-transfected cells (Figure 3.8B, 3.8C). Next, the requirement for COX-2 in AEA-induced apoptosis was examined. Apoptosis was significantly induced by AEA in cells transfected with COX-2 (Figure 3.8D and 3.8E). Taken together, these data show that in AEA-treated cells, COX-2 is required for ER stress-induced apoptosis.

3.3.8. J-series prostamide synthesis occurred in parallel with ER stress-apoptosis

COX-2 metabolizes AA to PGH₂, which is converted by PGDS to PGD₂; PGD₂ is then dehydrated to J-series prostaglandins. To confirm that this synthetic pathway is preserved for AEA-derived J-series prostamides, JWF2 keratinocytes were exposed to the selective COX-2 inhibitor, NS-398, prior to treatment with AEA and J-series prostamides were detected using LC-MS. The inhibition of COX-2 reduced J-series prostamides to low levels that were comparable to vehicle-treated cells (Figure 3.9A). To evaluate whether the blockade of J-series prostaglandin synthesis could prevent ER stress-mediated apoptosis, a PGDS inhibitor was utilized. Two distinct types of PGDS have been identified: lipocalin-PGDS (L-PGDS) and hematopoietic-PGDS (H-PGDS). Both H-PGDS and L-PGDS were expressed in untreated JWF2 cells (Figure 3.9B). Next, JWF2 cells were exposed to SeCl₄, an H-PGDS and L-PGDS inhibitor (Qu et al., 2006) followed by cell exposure to AEA. As anticipated, AEA significantly increased the production of D- and J-series prostaglandins (Figure 3.9C-D). Pretreatment of cells with SeCl₄, caused a significant reduction in AEA-mediated D- and J-series prostaglandin synthesis. In addition, SeCl₄ abrogated AEA-induced CHOP10 expression and caspase 3 cleavage (Figure 3.9E) indicating the necessity of PGDS metabolic products for ER stress-mediated apoptosis.

3.3.9. PGD₂-ethanolamide metabolism to J-series prostamides resulted in ER stress-mediated apoptosis

Synthetic J-series prostamides or selective inhibitors of J-series prostaglandin synthesis were not commercially available, therefore we could not directly determine if J-series prostamides mediated the cytotoxic effects of AEA. Therefore, cells were exposed to the J-series prostamide precursor PGD₂-EA and ER stress, apoptosis, and J-series prostaglandin

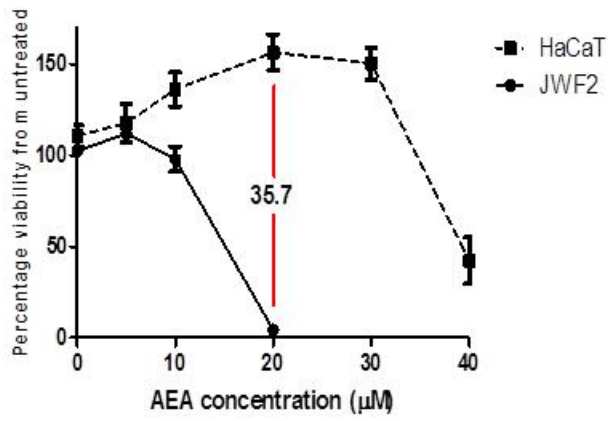
synthesis were measured. Figure 3.10A showed that 20 μ M PGD₂-EA increased the expression of CHOP10 and induced caspase-3 cleavage. LC-MS analysis of the culture medium from these experiments demonstrated that exogenously applied PGD₂-EA was metabolized to 15-deoxy $\Delta^{12,14}$ PGJ₂-EA (m/z 358) and that unconjugated 15-deoxy $\Delta^{12,14}$ PGJ₂ was not detected (Figure 3.10B). The identity of m/z 358 was confirmed by conducting MS/MS (Figure 3.10C). These results verify that D-series prostamide was metabolized to J-series prostamides that recapitulate the effects of AEA on ER stress-mediated apoptosis.

3.4. FIGURES

Figure 3.1: AEA was preferentially toxic to tumorigenic keratinocytes that overexpress COX-2

(A) Tumorigenic JWF2 and non-tumorigenic HaCaT keratinocytes were treated with different concentrations of AEA for 24 hours and cell viability was measured by conducting MTS assay. Fold difference in the viability of JWF2 and HaCaT cells treated with 20 μ M AEA was calculated. (B) Western blot analysis was conducted to compare FAAH and COX-2 expression in JWF2 and HaCaT keratinocytes. COX-2 levels were normalized to the corresponding GAPDH value producing an optical density of 0.0024 and 0.810 in HaCaT and JWF2 cells, respectively.

A.



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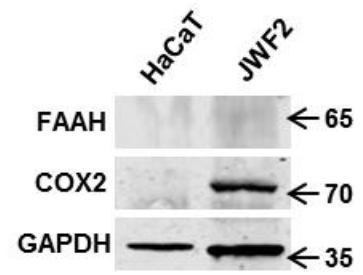
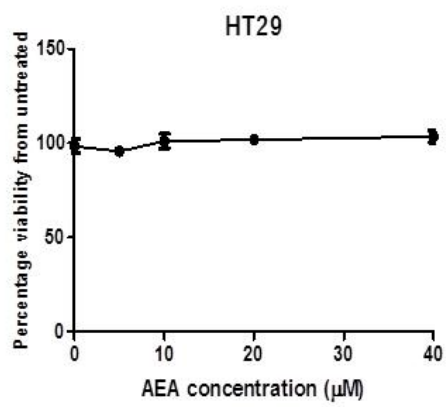


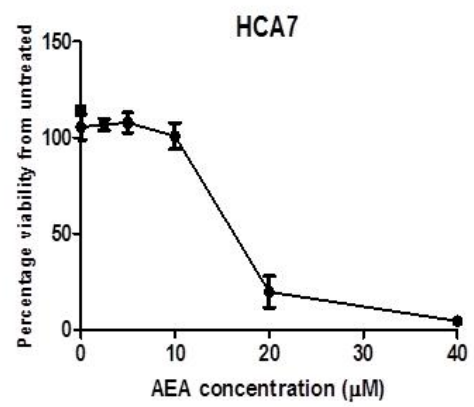
Figure 3.2: AEA induced cell death in tumor cell lines with high COX-2 and low FAAH expression

(A,B) Human colorectal carcinoma (HT29, A, and HCA7, B) were exposed to different concentration of AEA for 24 hours and cell viability was measured using MTS assay. (C) Western blot analysis was conducted to compare FAAH and COX-2 expression in HCA7 and HT29 cells. FAAH levels were normalized to the corresponding GAPDH value producing an optical density of 2.14 and 0.97 in HT29 and HCA7 cells, respectively. COX-2 expression produced an optical density of 0.27 and 1.2 in HT29 and HCA7 cells, respectively after normalization to the corresponding GAPDH value.

A.



B.



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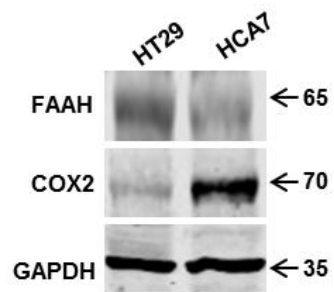


Figure 3.3: AEA caused ER stress in non-melanoma skin cancer cells

(A) JWF2 cells were treated with 20 Δ M AEA or vehicle [ETOH as a solvent control; \leq 0.1% (v/v) final concentration] for 2 hours. ATF6 (green fluorescence) or the nuclear membrane protein, lamin B, (blue fluorescence) were detected by confocal microscopy (left panels). Histogram (right panels) represents the nuclear fraction (nuclear ATF6/ total ATF6) quantified from confocal images. Data were analyzed using a t-test and are represented as mean \pm SEM (*, $P < 0.05$). (B) JWF2 cells were treated with 20 μ M AEA or vehicle for 1 hour and 2 hours. Cells were also treated with thapsigargin (TG) for 2 hours as positive control for ER stress induction. The levels of phospho-PERK (P-PERK), total PERK (t-PERK), phospho-eIF2 α (P-eIF2 α), total eIF2 α (t-eIF2 α), and GAPDH (loading control) were examined by Western blot analysis. Band intensities were quantified using ImageJ software. Fold induction in protein expression was determined by comparing the band intensities of samples to vehicle-treated cells after normalizing GAPDH levels. (C, Top panels) JWF2 cells were treated with 20 μ M AEA for 2 hours and 4 hours. Immunoblots show phospho-IRE1 (P-IRE1), total IRE1 (t-IRE1), and GAPDH. (C; Bottom panel) JWF2 cells were treated with AEA for 4 hours, mRNA isolated, and RT-PCR analysis was conducted. Negative control (NC) lane is RT-PCR reaction conducted in absence of mRNA. The marker used (M) is a low DNA Mass Ladder (Invitrogen). Unspliced (US) and spliced (S) XBP1 are shown. GAPDH mRNA levels were examined as a loading control. (D) JWF2 cells were treated with 20 μ M AEA or vehicle for different periods of time and J-series prostaglandins measured by ELISA. Data were analyzed using a t-test and are represented as mean \pm SEM (*, $P < 0.05$).

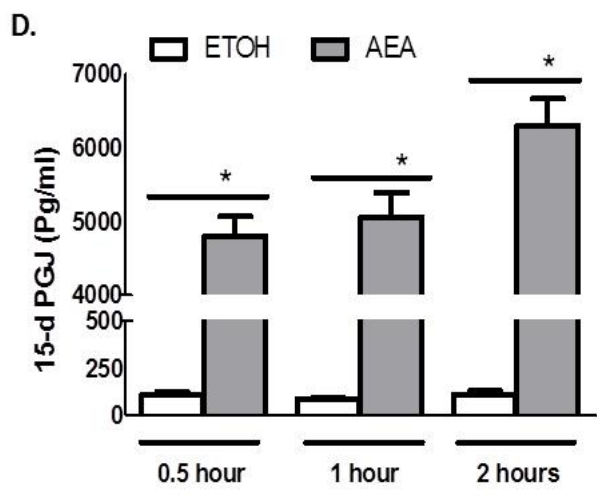
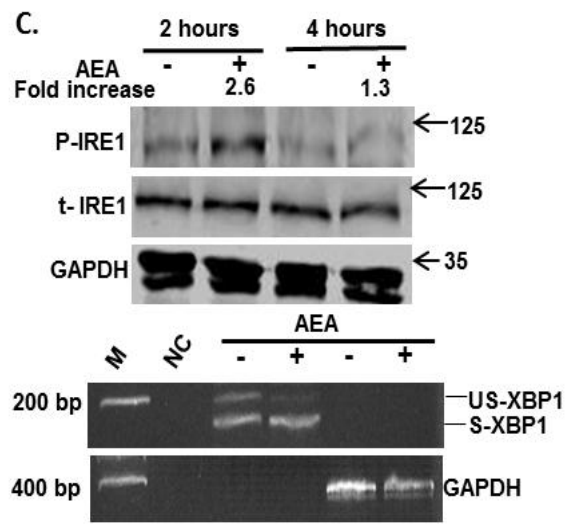
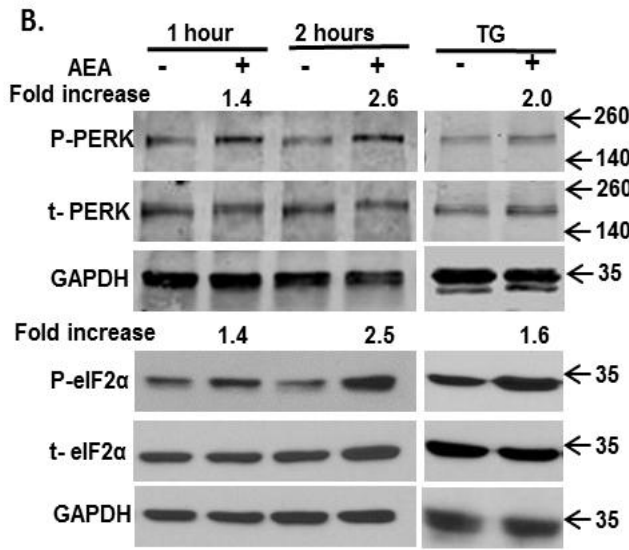
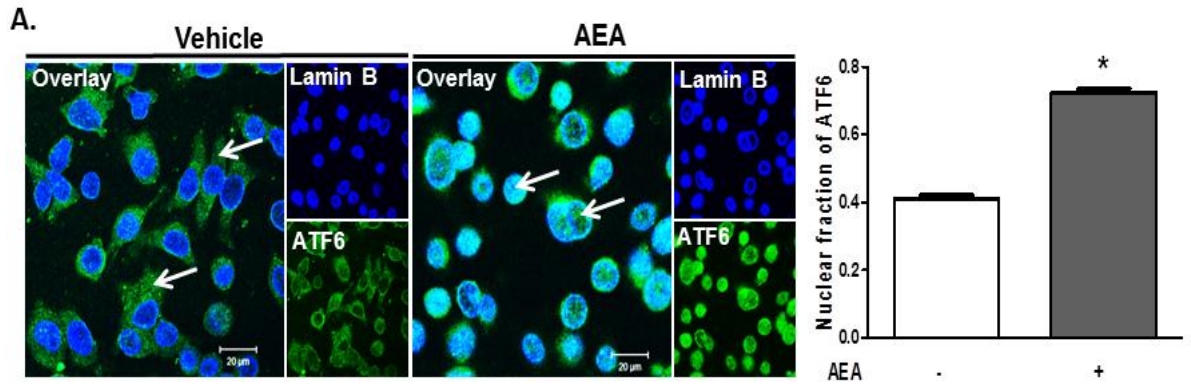
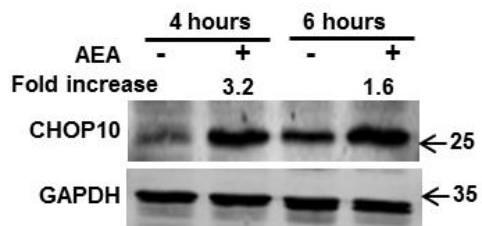


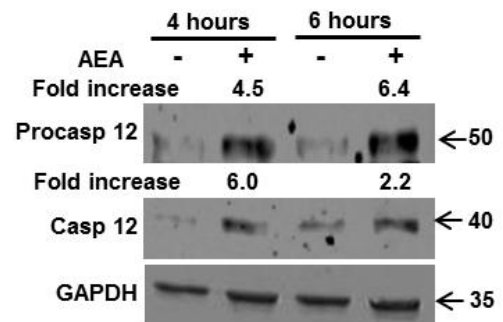
Figure 3.4: AEA activated ER stress-apoptotic signaling in non-melanoma skin cancer cells

(A-D) JWF2 cells were treated with 20 μ M AEA or vehicle for 4 and 6 hours. Western blot analysis was conducted to detect (A) CHOP10, (B) procaspase 12 and cleaved caspase 12, (C) full-length caspase 3 and cleaved caspase 3. GAPDH expression was used as loading control. (D) Histogram represents cleaved caspase 3 levels in JWF2 cells treated with AEA for 4 and 6 hours (represented in Figure 2C). The densitometric values of cleaved caspase 3 from three independent experiments were analyzed using t- tests and are displayed as mean \pm SEM (* indicates statistically significant difference between AEA-treated cells and vehicle-treated cells, $P < 0.05$).

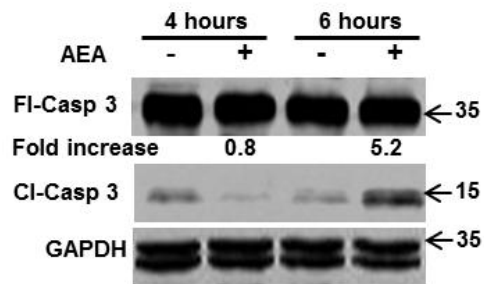
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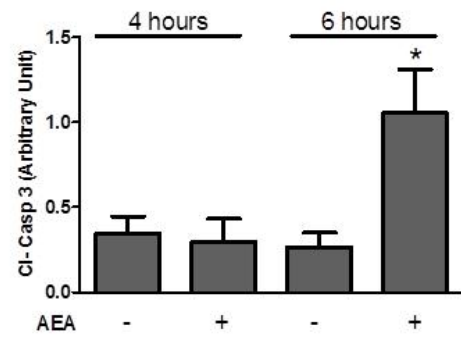


Figure 3.5 : ER stress was necessary for AEA-induced apoptosis in non-melanoma skin cancer cells

JWF2 cells were pretreated for 1 hour with 50 μ M salubrinal and then treated with 20 μ M AEA or vehicle. (A) Western blot analysis was conducted to examine P-eIF2 α and t-eIF2 α expression after 2 and 6 hours. (B) Cleaved caspase 3 was detected after 6 hours by conducting Western blot analysis. The fold increase in protein expression compared with vehicle-treated cells was determined using ImageJ software and is indicated above each figure. (C) Cell viability was determined using ImageJ software and is indicated above each figure. (C) Cell viability was measured after 8 hours by conducting MTS experiments. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* indicates statistically significant difference between the samples and vehicle-treated cells and # indicates statistically significant difference between the samples and cells treated with AEA alone, $P < 0.05$).

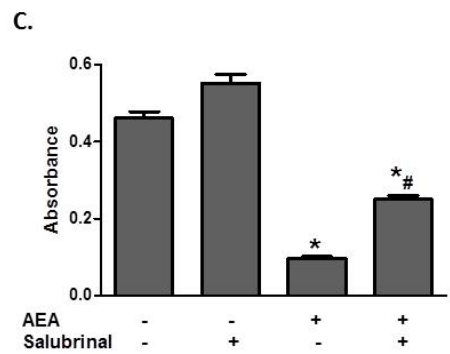
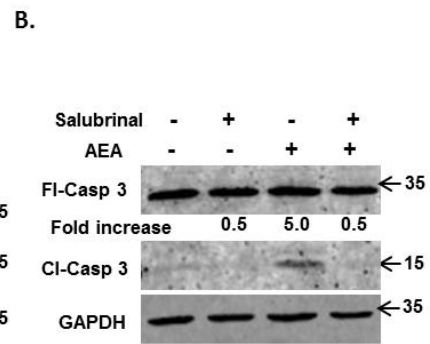
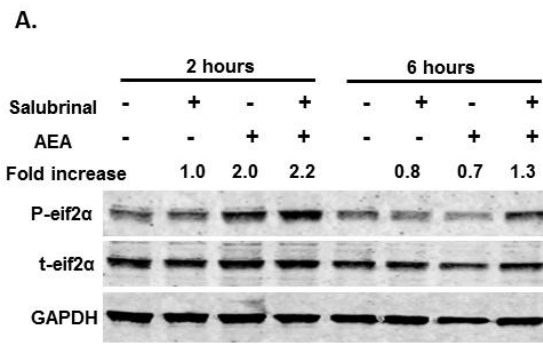


Figure 3.6: AEA was metabolized to novel J-series prostamides

(A, B) LC-MS/MS of peaks (A) m/z 358 and (B) m/z 376 was used to confirm identity of J-series prostamides in AEA-treated JWF2 cells. J-series prostamide signal was only observed in AEA-treated (insets, red), but not EtOH-treated (insets, blue) cells. Note PGJ₂-EA and Δ^{12} PGJ₂-EA are isomers (m/z 376) which co-elute from the LC and cannot be distinguished based on m/z. (C) LC-MS/MS at 5eV collision energy produced a peak at m/z 358, with an intensity that was much lower than the 15-deoxy $\Delta^{12,14}$ PGJ₂-EA metabolite peak observed in LC-MS spectra from JWF2 cells.

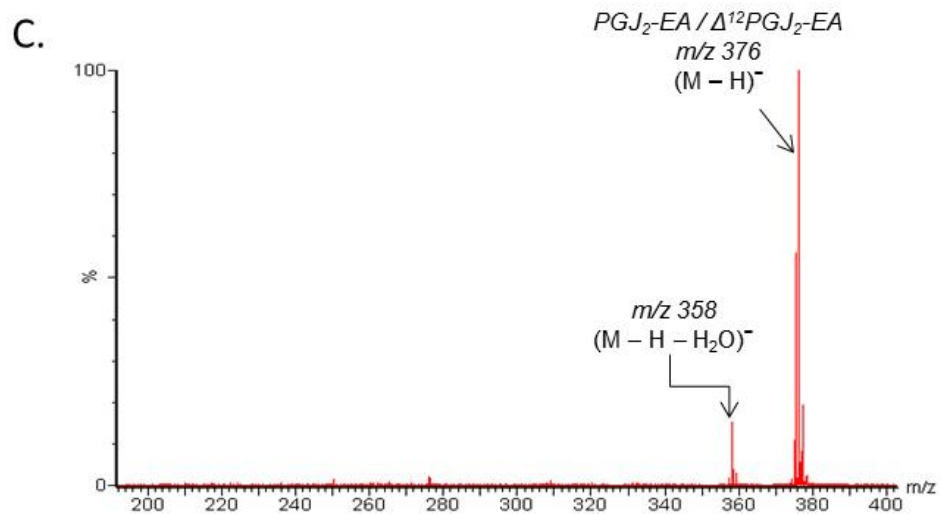
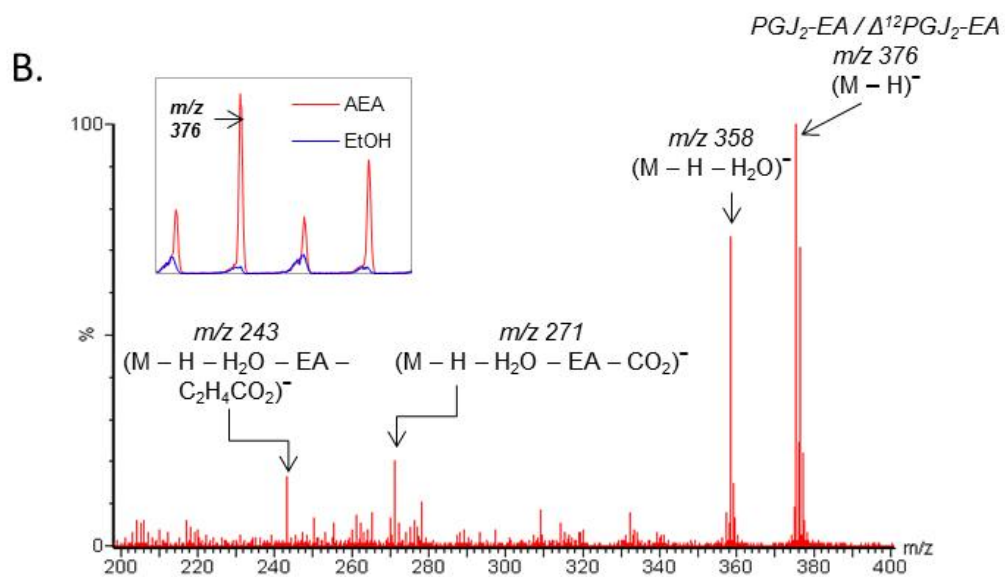
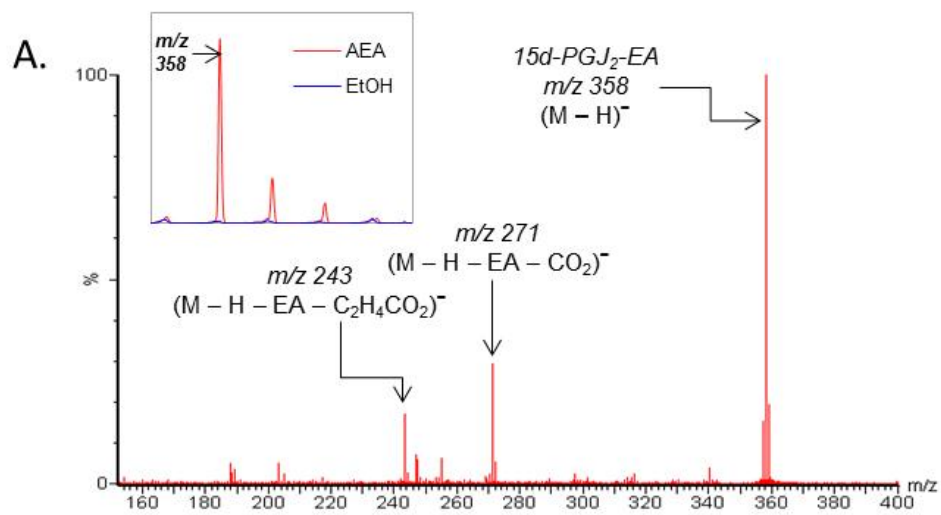


Figure 3.7 : AEA-induced ER stress was dependent on COX-2

HaCaT cells were transfected with PcDNA6.1-h-COX-2 (red fluorescence) or the empty vector, pcDNA6.1, and treated with 20 μ M AEA or vehicle for 2 hours. (A) P-eIF2 α (green fluorescence) was detected by confocal microscopy. (B) Histogram shows quantification of the intensity of P-eIF2 α fluorescence in all cells or only in cells that express COX-2 (inset). (C) ATF6 (green fluorescence) was detected by confocal microscopy. (D) Histogram shows quantification of the nuclear fraction of ATF6 in all cells or only in cells that express COX-2 (inset). Data were analyzed using ANOVA followed by Tukey's multiple comparison tests. Data are represented as mean +SEM (* represents statistically significant difference from vehicle-treated empty vector transfected cells and # represents statistically significant difference from AEA-treated empty vector transfected cells, $P < 0.05$). Data in inset were analyzed using a t-test and are represented as mean \pm SEM (*, $P < 0.05$).

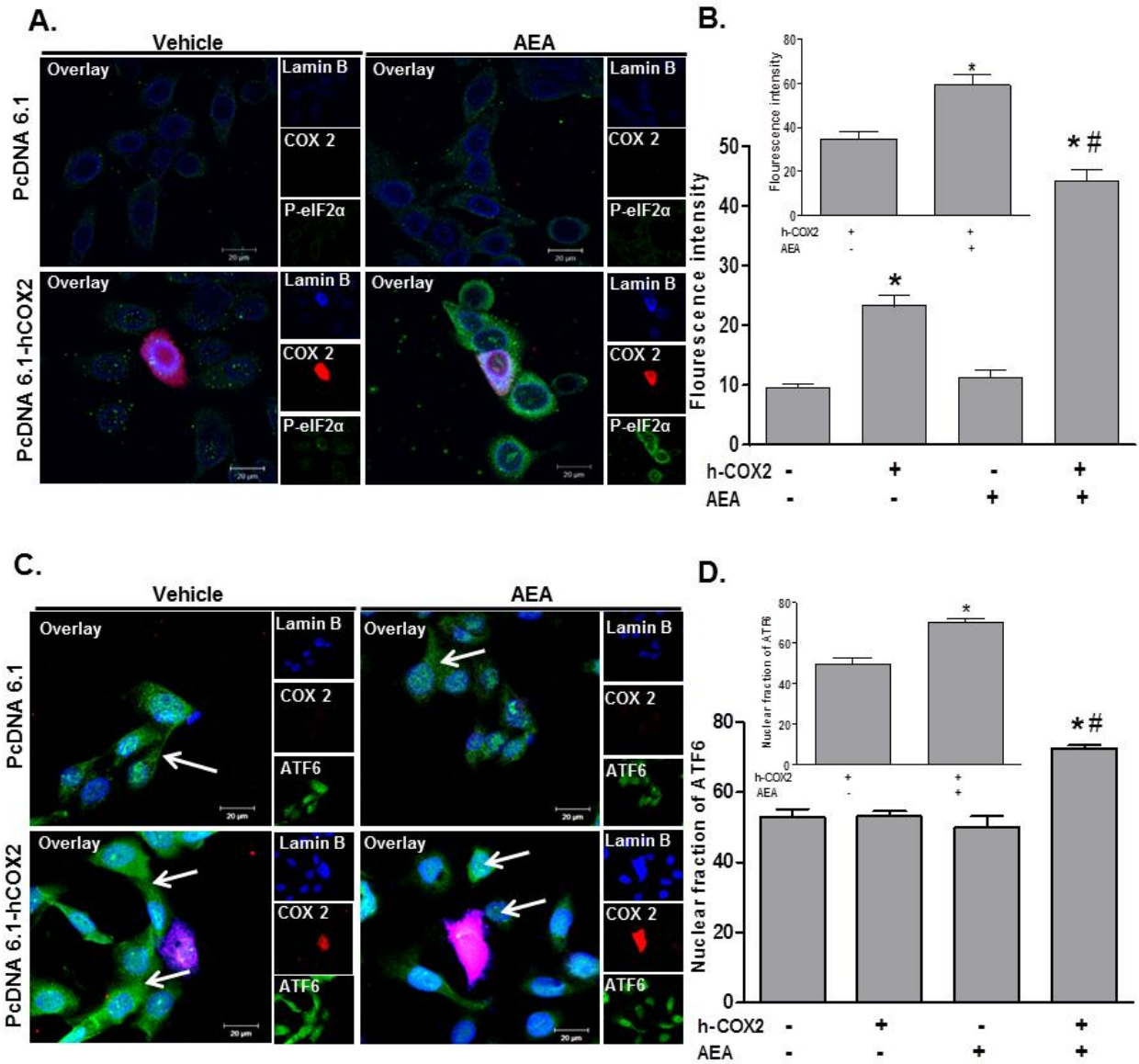
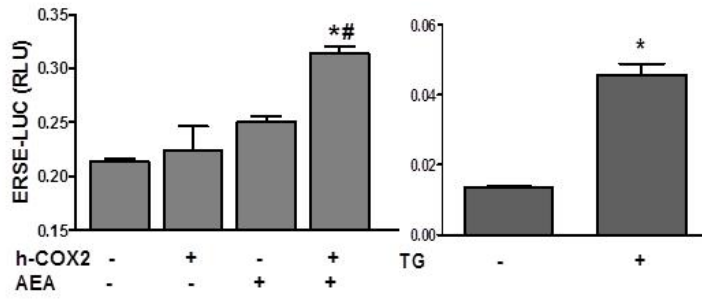


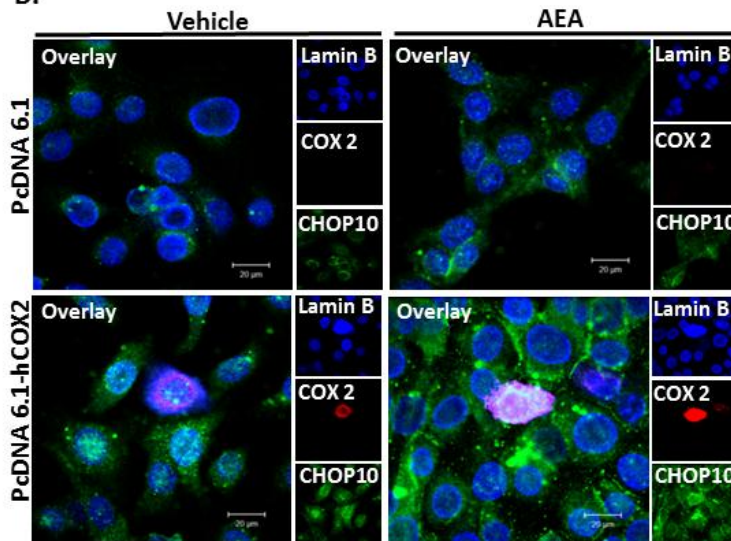
Figure 3.8: AEA-induced CHOP10 expression and apoptosis required COX-2

HaCaT cells were transfected with PcDNA-6.1-h-COX-2 or PcDNA6.1 (empty vector control) and treated with 20 μ M AEA or vehicle. (A) HaCaT cells were co-transfected with the luciferase-tagged ERSE reporter plus Renilla luciferase and treated with 20 μ M AEA or vehicle for 2 hours (left). ERSE-luciferase plus Renilla luciferase-transfected HaCaT cells were treated with thapsigargin (TG) as positive control (right). (B-E) HaCaT cells were transfected with PcDNA-6.1-h-COX-2 or PcDNA6.1 and treated with 20 μ M AEA or vehicle. (B) CHOP10 (green fluorescence) and COX-2 (red fluorescence) expression were detected after 7 hours by conducting confocal microscopic analysis. (C) Histogram shows quantification of the intensity of CHOP10 fluorescence. Data were analyzed using ANOVA followed by Tukey's multiple comparison test. Data are represented as mean \pm SEM (* represents statistically significant difference from vehicle-treated empty vector transfected cells and # represents statistically significant difference from AEA-treated empty vector transfected cells, $P < 0.05$). The inset shows CHOP10 intensity only in cells that express COX-2. Data in inset were analyzed using a t-test and are represented as mean \pm SEM (*, $P < 0.05$). (D) Activated caspase-3 (green fluorescence) and COX-2 (red fluorescence) were detected after 10 hours using confocal microscopy. (E) Histogram represents the number of COX-2 (red fluorescence) and activated Casp-3 (green fluorescence) expressing cells measured as a fraction of the total number of cells in the same field. Data were analyzed using ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* represents statistically significant difference from vehicle-treated empty vector transfected cells and # represents statistically significant difference from AEA-treated empty vector transfected cells, $P < 0.05$).

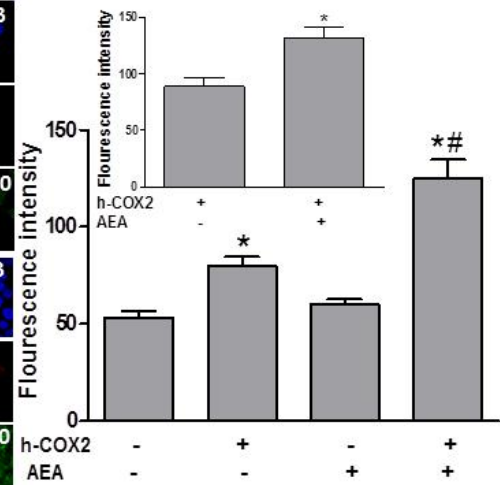
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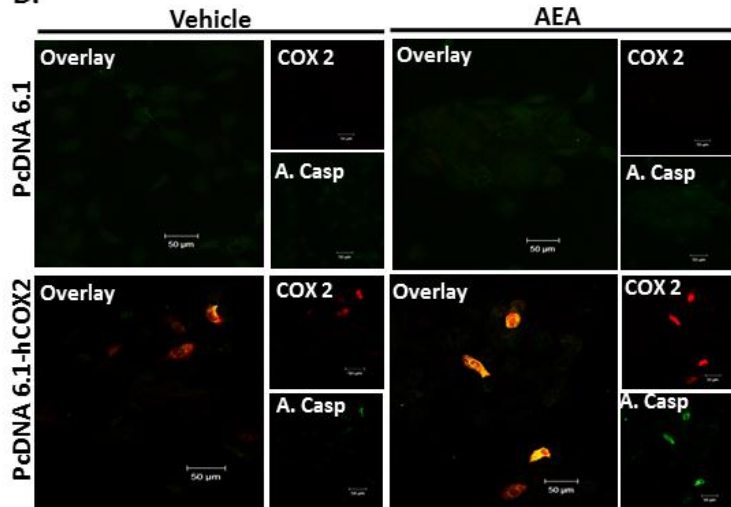
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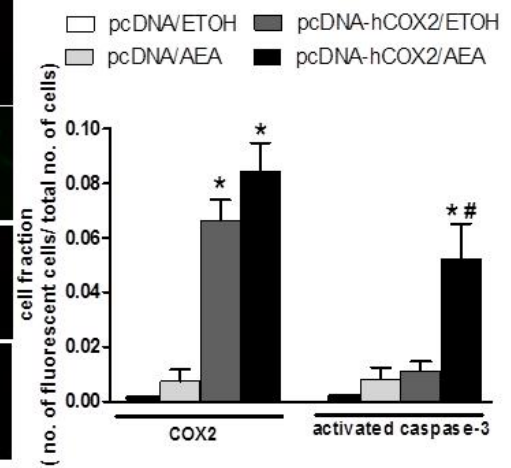


Figure 3.9: AEA-derived J-series prostaglandins were required for ER stress and apoptosis

(A) JWF2 cells were treated with COX-2 inhibitor, NS-398, followed by AEA (20 μ M) or vehicle for 5 hours. PGJ₂-EA/ Δ^{12} PGJ₂-EA (m/z 376) and 15-deoxy $\Delta^{12,14}$ PGJ₂-EA (m/z 358) that accumulated in the media were detected by conducting mass spectrometry analysis. (B) Hematopoietic and Lipocalin PGDS expression was examined in untreated JWF2 cells (duplicate samples) by conducting Western blot analysis. GAPDH expression was used as loading control. (C-D) JWF2 cells were pretreated with SeCL4 and then treated with 20 μ M AEA or vehicle for 4 hours. ELISA analysis was conducted to measure D-series prostaglandins (C) and J-series prostaglandins (D) as directed by the manufacturers. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* indicates statistically significant difference between the samples and vehicle-treated cells and # indicates statistically significant difference between the samples and cells treated with AEA alone, P<0.05). (E) CHOP10 and cleaved caspase 3 levels were examined in cells pretreated with 100 μ M SeCL4 and treated with 20 μ M AEA or vehicle by conducting Western blot analysis. The fold increase in protein expression compared with vehicle-treated cells is shown above the figure.

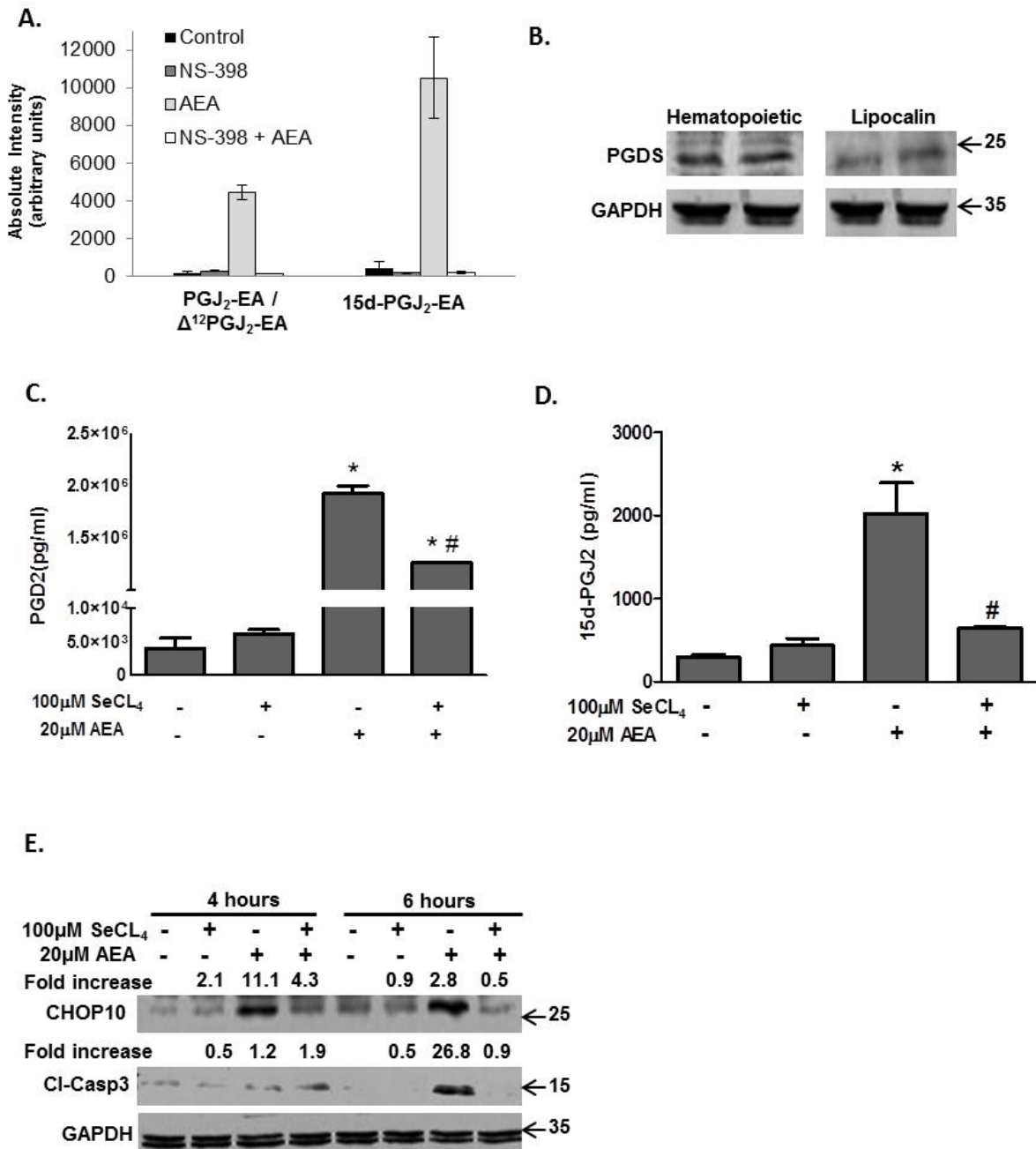
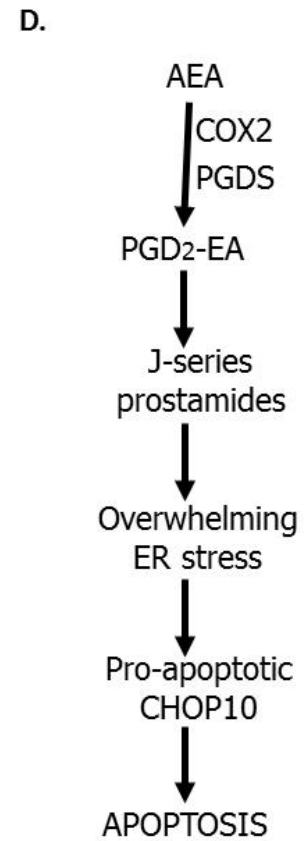
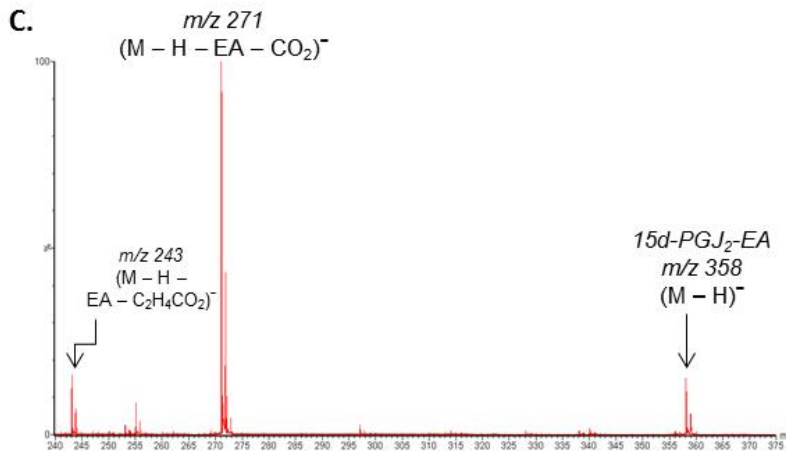
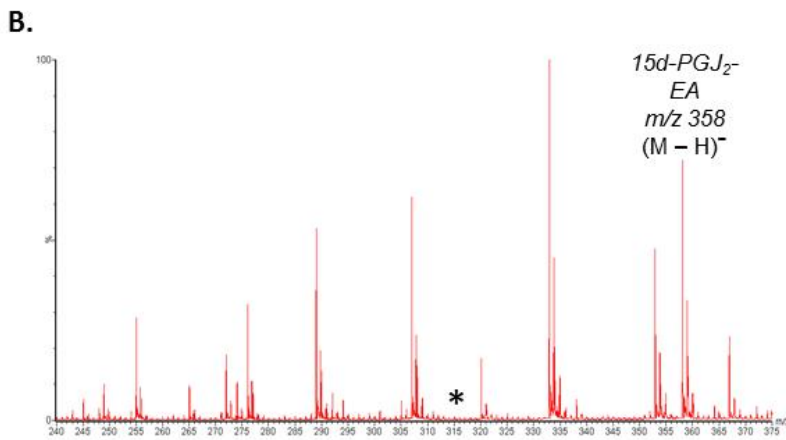
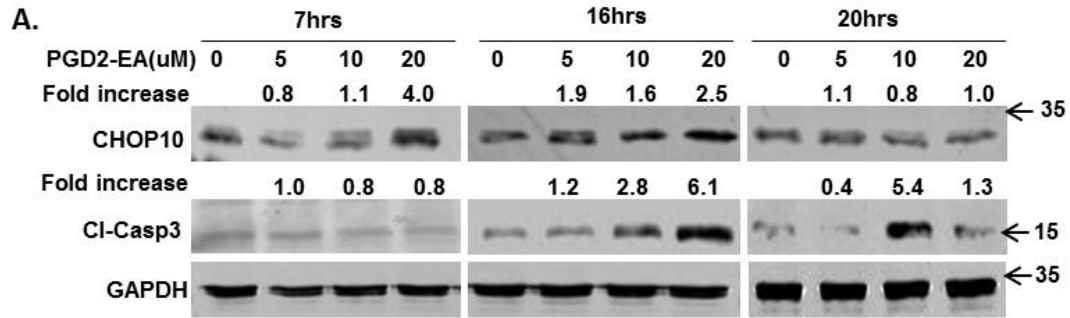


Figure 3.10: PGD₂-EA was metabolized to J-series prostamides that increased CHOP10 and apoptosis

JWF2 cells were treated with 5, 10 and 20 μ M PGD₂-EA or vehicle for 7, 16 and 20 hours. (A) The expression of CHOP10 and cleaved caspase 3 was examined by conducting Western blot analysis. Culture medium from experiments in (A) was collected after 7 hours, prostaglandins extracted and (B) LC-MS was conducted to detect 15-deoxy $\Delta^{12,14}$ PGJ₂-EA (m/z 358). 15d-PGJ₂ (m/z 315,*) is not detected. (C) The identity of 15-deoxy $\Delta^{12,14}$ PGJ₂-EA was confirmed with LC-MS/MS. (D) Proposed pathway of AEA-induced apoptosis. We propose that in tumor cells which overexpress COX-2, AEA is metabolized by COX-2 followed by PGDS to J-series prostamides. J-series prostamides then induce overwhelming ER stress which increases CHOP10 expression and initiates the apoptotic cascade.



3.5. DISCUSSION

Gaining an understanding of the molecular mechanisms underlying AEA-induced apoptosis in tumor cells is a topic of increasing interest. In the present report, we determined that AEA was selectively toxic to COX-2 overexpressing cells. AEA also caused ER stress and that signaling through the ER stress pathway was necessary for AEA-induced apoptosis. In addition, AEA-stimulated ER stress and apoptosis only occurred in the presence of COX-2. Moreover, we demonstrated for the first time that AEA was metabolized by COX-2 to the novel J-series prostamides, PGJ₂-EA, Δ¹²PGJ₂-EA, and 15-deoxyΔ^{12,14}PGJ₂-EA. Furthermore, J-series prostamide synthesis was required for ER stress-associated apoptosis initiated by AEA. Hence, the results of this study support our hypothesis that AEA is metabolized by COX-2 to J-series prostamides that activate apoptotic ER stress (Figure 3.10D).

Several groups have demonstrated that COX-2 activity is necessary for AEA-induced cell death. For example, AEA and its analog, R(+)-methanandamide caused COX-2 dependent cell death in colorectal carcinoma and neuroglioma cells, respectively (Ramer et al., 2001; Patsos et al., 2010). Furthermore, AEA-initiated apoptosis was partially mediated by COX-2 in embryonal carcinoma cells (47). In the current study, we demonstrated that COX-2 was necessary for apoptosis mediated by AEA (Figure 3.8). A few of the above mentioned reports specifically identify COX-2 metabolic products of AEA as mediators of apoptosis. However, since AEA is also metabolized by enzymes including 12-lipoxygenase (Edgemond et al., 1998) and P450 enzymes (Snider et al., 2009), it is possible that these endogenous enzymes play a role in AEA-induced ER stress and apoptosis. The data presented here however, argue against the idea that AEA-mediated apoptosis is caused by lipoxygenases, P45Os, or other enzymes since we did not observe ER stress-associated apoptosis in AEA-treated cells that lacked COX-2

expression (Figure 3.7 and Figure 3.8, AEA-treated, empty vector transfected cells). Thus, COX-2 is a prominent regulator of AEA-mediated apoptosis.

Since AEA-induced cell death is dependent on COX-2, it is reasonable to propose that COX-2 promotes apoptosis by converting AEA to J-series prostamides. Our data showed that novel J-series prostamides were synthesized in AEA-treated cells that overexpress COX-2 (Figure 3.6). In addition, blockade of J-series prostamide synthesis using a PGDS inhibitor prevented AEA-induced ER stress and apoptosis (Figure 3.9). Moreover, exogenous PGD₂-EA was converted to J-series prostamides which mimicked the effects of AEA by inducing ER stress-apoptosis (Figure 3.10A-C). Furthermore, an earlier report by our group demonstrated that only exogenous D- and J-series prostaglandins (but not E- and F-type prostaglandins) were capable of inducing cell death in tumorigenic keratinocytes. Thus, it appears that AEA-induced ER stress-apoptosis is mediated by J-series prostamides. Unfortunately, it was not possible to directly test whether J-series prostamides initiated ER stress-mediated apoptosis since these bioactive lipids or their specific inhibitors were not commercially available. However, various groups have demonstrated that J-series prostaglandins that are derived from AA cause apoptosis by different cellular mechanisms. It has been reported that 15-deoxy $\Delta^{12,14}$ PGJ₂ activated the apoptotic cascade via agonism of PPAR-gamma (Clay et al., 1999). 15-deoxy $\Delta^{12,14}$ PGJ₂ also induced apoptosis in HCT-116 cells by activating the ER stress pathway (Su et al., 2008). Hence, the proposal that J-series prostamides are ultimately responsible for AEA-induced ER stress and apoptosis is consistent with the data presented here and the known effects of arachidonic acid-derived J-series PGs.

Whether J-series prostaglandins induce tumor cell apoptosis *in vivo* is controversial. A study by Clay et al. reported that AA-derived 15-deoxy $\Delta^{12,14}$ PGJ₂ activated the apoptotic cascade

in MDA-MB-231 breast cancer cells and prevented breast cancer cell tumorigenesis (Clay et al., 1999). In contrast, Millan et al. found that 15-deoxy $\Delta^{12,14}$ PGJ₂ increased DMBA/TPA-mediated skin tumor development when applied topically during tumor initiation (Millan et al., 2006). These opposing effects might be due to the drug dosage used in each study as J-series prostaglandins, like most pharmacological agents, produce biphasic effects (Levonen et al., 2001). Thus, it is possible that J-series prostamides will promote tumor cell death similar to J-series prostaglandins under optimal experimental conditions. However, it is premature to assume that J-series prostamides will exhibit the same effects as its AA-derived counterpart. A study by Matias et al., showed that AEA-derived prostamides (PGE₂-EA, PGF_{2 α} -EA, and PGD₂-EA) elicit cat iris contraction independent of EP-, FP- and DP-receptors unlike AA-derived prostaglandins (PGE₂, PGF_{2 α} , and PGD₂) (Matias et al., 2004). Although cell surface receptors for J-series prostaglandins have not been identified, the report by Matias suggests that the effects of AEA-derived J-series prostamides may be distinct from arachidonic acid-derived, J-series prostaglandins. To gain a better understanding of its activity *in vitro* and *in vivo*, J-series prostamides have been synthesized and are being characterized by our group.

AEA-induced death is regulated by several cell signaling pathways. The induction of cell death by AEA was reported to be dependent upon the stimulation of ceramide synthesis and Fas/FasL localization to lipid rafts in cholangiocarcinoma cells (DeMorrow et al., 2007). In addition, AEA-induced neuroblastoma apoptosis was prevented by TRPV1 channel inhibition through a calcium sensitive mechanism (Maccarrone et al., 2000). Also, AEA cytotoxicity was found to be regulated by the activity of enzymes that promote endocannabinoid degradation (Bifulco et al., 2004; Siegmund et al., 2006; Kuc et al., 2012). Our data suggest that ER stress is another signaling pathway that regulates AEA-induced apoptosis. We found that AEA

stimulated the expression and activity of multiple ER stress proteins and also induced apoptosis (Figure 3.3 and 3.4). In addition, AEA-induced cell death was partially reversed by interfering with ER stress signaling (Figure 3.5 A-C). Consistent with these results, other groups have demonstrated that ER stress regulates cannabinoid-induced cell death. For instance, in neuroblastoma cells AEA-mediated apoptosis was blocked by disabling the ER stress protein BiP (Pasquariello et al., 2009). Carracedo et al. showed that apoptosis caused by Δ^9 tetrahydrocannabinol (Δ^9 -THC) required CB2R and ceramide-induced ER stress in pancreatic tumor cells (Carracedo et al., 2006). Also, J-series prostaglandins that are derived from AA initiate apoptosis via the ER stress pathway (Su et al., 2008). Furthermore, it has been demonstrated that synthetic prostaglandins with structures similar to J-series prostaglandins accumulate in the ER and trigger activation of BiP (Takahashi et al., 1998). Thus, the endocannabinoid, AEA, and its J-series prostamide metabolic products, likely initiate apoptosis by inducing ER stress.

The molecular events required for activation of the apoptotic ER stress pathway are unclear but a few important studies are beginning to shed light on this issue. Lin et al demonstrated that the initiation of apoptotic ER stress required continuous PERK signaling accompanied by the activation and subsequent deactivation of IRE1 and ATF6 in human embryonic kidney cells (Lin et al., 2007). Moreover, this group demonstrated that increasing IRE1 expression under these conditions prevented cell death indicating that IRE1 inactivation was necessary for apoptosis. Another study determined that under low levels of ER stress the endonuclease activity of IRE1 promoted splicing of XBP1 and cell survival. However, intense ER stress initiated global splicing of mRNAs present within the ER and triggered the apoptotic cascade in insulinoma cells (Han et al., 2009). In addition, Su et al determined that the magnitude

of proapoptotic CHOP10 expression was a critical determinant of apoptotic ER stress in HepG2 cells as maximal but not moderate levels of CHOP10 caused ER stress-mediated apoptosis (Su and Kilberg, 2008). Our data show that some elements of these pathways are preserved in tumorigenic keratinocytes treated with AEA. Specifically, we found IRE1 was activated and then deactivated in JWF2 keratinocytes (Figure 3.3C) similar to the findings of Lin et al. Also, AEA caused high level CHOP10 expression and apoptosis in HaCaT cells with ectopic COX-2 expression however, in the absence of COX-2, AEA-induced CHOP10 levels were low and apoptosis did not occur (Figure 3.8). Thus, it is tempting to speculate that IRE1 deactivation and elevated CHOP10 levels were essential triggers of ER-stress apoptosis in keratinocytes treated with AEA. Given the diversity of pathways that have been shown to activate apoptosis, cytotoxic ER stress likely occurs in a cell type- and context-specific manner. Future studies with AEA will seek to identify the ER stress pathway proteins that are needed to initiate apoptosis in tumorigenic epithelial cells that overexpress COX-2.

The data presented in this dissertation show that AEA is cytotoxic to cells that overexpress COX-2. We have identified novel J-series prostamides as likely regulators of apoptosis, which our data implies was mediated by ER stress. The requirement for COX-2 in AEA-induced ER stress-apoptosis suggests that rather than inhibiting COX-2 as a therapeutic strategy, we can take advantage of COX-2 overexpression in tumor cells to metabolize AEA to these cytotoxic J-series prostamides. Since the endogenous levels of COX-2 are low in non-tumor keratinocytes, AEA may produce selective toxicity in tumor cells and provide an effective approach for topical treatment of NMSC or other cancers that overexpress COX-2.

**CHAPTER FOUR: ANANDAMIDE-INDUCED APOPTOSIS IN NON-MELANOMA
SKIN CANCER IS MEDIATED BY OXIDATIVE STRESS IN A RECEPTOR-
INDEPENDENT MANNER**

4.1. ABSTRACT

Endocannabinoids are neuromodulatory lipids that regulate central and peripheral physiological functions. Endocannabinoids have emerged as effective antitumor drugs due to their ability to induce growth arrest and apoptosis in a series of cancer studies. In several reports, the antiproliferative activity of these biological lipids was mediated by the G-protein coupled receptors, CB1 and CB2. However, recent studies have demonstrated that receptor-independent effects may also account for their activity. Our previous studies reported that the antiproliferative activity of anandamide (aka arachidonyl ethanolamide, AEA) was regulated by COX-2 via the ER stress pathway. In addition, we determined that AEA induced oxidative stress. Moreover, AEA-induced cell death occurred by a cannabinoid receptor-independent mechanism. However, it was unclear if oxidative stress was required for apoptosis and if oxidative and ER stress occurred in a receptor-dependent or -independent manner. Therefore, the current study examines the role of oxidative stress in the induction of ER stress-mediated apoptosis and investigates whether this effect is caused by CB1 and/or CB2 receptors. In the non-melanoma skin cancer (NMSC) cell line, JWF2, AEA reduced the total intracellular level of glutathione and induced oxidative stress. To evaluate the importance of oxidative stress in AEA-induced cell death, N-acetyl cysteine (NAC) and Trolox antioxidants were used. The two antioxidants ameliorated the antiproliferative effect of AEA. Furthermore, Trolox inhibited AEA-induced CHOP10 expression and caspase 3 cleavage, indicating that oxidative stress is required for AEA-induced ER stress-apoptosis. On the other hand, selective blockade of the CB1 and CB2 receptors did not

inhibit AEA-induced oxidative stress, ER stress or apoptosis. These findings suggest that AEA-induced ER stress-apoptosis in NMSCs is mediated by oxidative stress through a receptor-independent mechanism. Hence, receptor-independent AEA signaling pathway may be targeted to eliminate NMSC cells.

4.2. INTRODUCTION

Cannabinoids are a diverse class of compounds that regulate physiologic and pathologic processes including the appetite, mood, learning/memory, pain sensation and inflammation. Cannabinoids have been utilized clinically and in animal studies to prevent the adverse effects associated with cancer chemotherapy. The cannabinoids dronabilone and nabilone are clinically approved to prevent chemotherapy-induced nausea and vomiting (Tramer et al., 2001; Alexander et al., 2009). Also, the cannabinoid N-arachidonoyl ethanolamide [(AEA); also known as anandamide], reduced pain and neurotoxicity caused by the cancer chemotherapeutic agent, cisplatin in an animal pain model (Khasabova et al., 2012). Many reports indicate that cannabinoids are also potent antineoplastic agents. It has been reported that these agents promote tumor cell death by initiating growth arrest (Fowler et al., 2003;Laezza et al., 2006;Frampton et al., 2010;Linsalata et al., 2010), inducing apoptosis and necrosis (Miyato et al., 2009;Wu et al., 2010;Patsos et al., 2010), and inhibiting angiogenesis and metastasis (Grimaldi et al., 2006;Pisanti et al., 2007;Laezza et al., 2008).

The biological effects of the endogenously synthesized cannabinoid (endocannabinoid), AEA, are primarily mediated by its interaction with cannabinoid receptors. The two classical cannabinoid receptor subtypes are cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). Both cannabinoid receptors are G-protein coupled and transduce signals through the $G_{i/o}$ protein (Das et al., 1995). Numerous studies demonstrate that cannabinoid receptor activation is required for the antineoplastic activity of AEA in some but not all tumor cells (Cianchi et al., 2008). AEA-mediated inhibition of cell proliferation was blocked by a selective CB1 receptor antagonist in breast and prostate cancer cell lines (De et al., 1998;Melck et al., 1999;Melck et al., 2000;Nithipatikom et al., 2011). In contrast, the induction of apoptosis by AEA was not

prevented by CB1 and CB2 receptor antagonists in various colon cancer cell lines (Gustafsson et al., 2009;Patsos et al., 2010) indicating that cannabinoid receptor modulation of tumor growth may be cell type or context dependent.

The cannabinoid receptor-independent antineoplastic activity of AEA is regulated by diverse molecular pathways. In cholangiosarcoma cells, AEA-induced cell death was mediated by increased ceramide production and Fas/FasL localization to lipid rafts and the use of CB1 or CB2 receptor antagonists did not inhibit the antiproliferative effect of AEA. (DeMorrow et al., 2007). In addition, AEA-induced apoptotic cell death in colon and skin cancer cell lines required COX-2 (Patsos et al., 2010;Soliman et al., 2014). COX-2 is an enzyme that promotes tumor growth and is overexpressed in different malignancies such as colon, breast, lung, and skin cancer. COX-2 metabolizes arachidonic acid to prostaglandins including PGE₂ or 15deoxy, $\Delta^{12,14}$ PGJ₂ (15d-PGJ₂) which initiates tumor cell growth or apoptosis, respectively. The anti-tumor effects of J series prostaglandins were mediated by oxidative stress in several reports (Takahashi et al., 1998;Saito et al., 2003;Weber et al., 2004;Chambers et al., 2007;Wang and Mak, 2011). Oxidative stress is caused by an imbalance in the production of cellular oxidants and their neutralization by enzymatic and non-enzymatic antioxidants in a cell (Mates et al., 2012). The most important endogenous non-enzymatic antioxidant is glutathione (GSH), a thiol-containing compound that maintains the intracellular redox balance. The reduction of GSH levels shifts the redox balance towards cellular oxidants and causes oxidative stress (Kondo et al., 2001). The overabundance of oxidative molecules in the cell causes damage to DNA, proteins and lipids and often leads to cell death (Kaur et al., 2014). Oxidative stress is also an important initiator of endoplasmic reticulum (ER) stress (Malhotra and Kaufman, 2007;Verfaillie et al., 2012;Niforou et al., 2014). ER stress occurs when stressors such as oxidative stress, increased

protein folding loads and calcium imbalance interfere with the cells ability to properly fold proteins leading to an accumulation of unfolded proteins and initiation of the unfolded protein response (UPR). Low levels of ER stress are resolved by activation of the ER sensors PERK, ATF6, and IRE1. Excessive or prolonged ER stress then triggers the expression of cytotoxic ER stress protein, CHOP-10 and ultimately initiates apoptosis (Lin et al., 2008;Chakrabarti et al., 2011). Previous studies from our group showed that the endocannabinoid, AEA selectively induces death in tumor cells in a COX-2-dependent and cannabinoid receptor-independent manner. In addition, we determined that ER stress and oxidative stress were required for AEA-induced apoptosis (Kuc et al., 2012;Soliman et al., 2014). However, the role of oxidative stress and the cannabinoid receptors in AEA-induced ER stress-apoptosis was unclear. The present study was undertaken to determine whether oxidative stress was essential for AEA-induced ER stress and apoptosis. Moreover, we examined whether AEA-induced apoptosis, oxidative stress, or ER stress are mediated by CB receptors. The results of our work suggest that in tumor cells which overexpress COX-2, AEA generates oxidative stress which activates the cytotoxic ER stress pathway in a cannabinoid receptor-independent manner.

4.3. RESULTS

4.3.1. AEA induced oxidative stress, ER stress and apoptosis in NMSCs

To confirm our previous finding that AEA induces cell death in NMSC cells, JWF2 keratinocytes were treated with different concentrations of AEA or vehicle for 8 hours and cell viability was measured using MTS assays. A significant reduction in cell viability was observed in cells treated with 10 - 40 μ M AEA (Figure 4.1A). To validate that AEA-induced cell death occurred via ER stress-mediated apoptosis, cells were treated with 20 μ M AEA and Western blot analysis was conducted to detect the expression of CHOP10, a marker for cytotoxic ER stress and cleaved caspase 3, an indicator of apoptosis (Figure 4.1B). CHOP10 expression and caspase 3 cleavage were stimulated by AEA in JWF2 cells confirming that cell death is mediated by ER stress-apoptosis.

We previously showed that AEA is metabolized by COX-2 to 15d-PGJ₂-EA and that this novel prostaglandin is likely responsible for the cytotoxic activity of AEA. Other groups have shown that COX-2 also metabolizes arachidonic acid to 15d-PGJ₂. Several studies indicate that 15d-PGJ₂ causes cell death by inducing oxidative stress (Kondo et al., 2001; Uchida and Shibata, 2008). However, since 15d-PGJ₂-EA was not commercially available and 15d-PGJ₂-EA and 15d-PGJ₂ are structurally similar, 15d-PGJ₂ was used to investigate whether J-series prostaglandins induce oxidative stress in tumorigenic keratinocytes. JWF2 cells were treated with AEA or 15d-PGJ₂ in the presence of the oxidative stress indicator, CM-H₂DCFDA and flow cytometric analysis was conducted. AEA caused a concentration-dependent increase in DCF oxidation (Figure 4.1C). 15d-PGJ₂ also significantly increased DCF fluorescence relative to vehicle-treated cells. In addition, AEA and 15d-PGJ₂ produced greater DCF oxidation in JWF2 cells than H₂O₂ which was used as a positive control. Collectively, these data indicate that AEA

is a potent inducer of oxidative stress and that the J-series metabolite of AEA could be responsible for this activity.

It has been demonstrated previously that a mechanism by which the arachidonic acid metabolite, 15d-PGJ₂, causes oxidative stress is by reducing total intracellular glutathione (GSH) levels (Levonen et al., 2001; Wang and Mak, 2011). To determine if AEA also decreases GSH, JWF2 cells were exposed to AEA, 15d-PGJ₂ or vehicle and then glutathione levels were measured. Figure 4.1D shows that the concentration of glutathione was significantly reduced by 20 μM AEA and 5 μM 15d-PGJ₂ suggesting that the cells capacity to rebound from this oxidative state may be compromised. Next, JWF2 cells were treated with AEA, the cells were divided, and flow cytometric analysis was conducted to detect oxidative stress (CM-H₂DCFDA) or ER stress (phosphorylated eIF2α). The mean fluorescence intensity of DCF (Figure 4.1E) and phospho-eIF2α (Figure 4.1F) were markedly increased indicating that both oxidative- and ER-stress occur and play an important role in AEA-induced apoptosis.

4.3.2. Oxidative stress was needed for the initiation of ER stress-apoptosis

To determine if oxidative stress was essential for AEA cytotoxicity, the intracellular oxidative environment was neutralized using the antioxidants, N-acetyl cysteine (NAC) and Trolox. NAC is a precursor of glutathione, which binds to and inactivates reactive molecules (Zafarullah et al., 2003). JWF2 keratinocytes were preincubated with different concentrations of NAC, treated with AEA, and CM-H₂DCFDA oxidation measured. NAC prevented AEA-induced oxidative stress in a concentration-dependent manner (Figure 4.2A). To evaluate whether a reduction in intracellular glutathione levels may have contributed to AEA-induced oxidative stress, total glutathione was measured. NAC restored the endogenous glutathione levels in AEA-treated cells (Figure 4.2B). A similar effect on AEA-induced oxidative stress was

observed when JWF2 cells were pretreated with Trolox (Figure 4.2C and 4.2D). Trolox is a water-soluble analog of vitamin E and exhibits antioxidant activity by scavenging free radicals (Mazor et al., 2006). Trolox (0.5 mM) completely inhibited AEA-induced oxidative stress although a significant restoration of endogenous glutathione levels was not seen. Furthermore, a reversal of AEA-induced cell death was seen in cells treated with NAC (Figure 4.2E) and partial reversal was seen with Trolox (Figure 4.2F).

The thiol group in NAC likely interacts directly with the double bond in the cyclopentanone ring of 15d-PGJ₂ leading to inactivation of 15d-PGJ₂. Therefore, the inhibition of AEA-induced cell death and oxidative stress shown in Figure 4.2A and 4.2E may have been caused by sequestration of the AEA metabolite, 15d-PGJ₂-EA, rather than by global modulation of the redox environment. As such, Trolox was utilized to determine the role of oxidative stress in AEA-induced ER stress and apoptosis. Trolox inhibited CHOP10 protein expression induced by AEA (Figure 4.3A). In addition, the data show that apoptosis mediated by AEA was prevented with Trolox (Figure 4.3B). These findings indicate that the generation of oxidative stress was important for the initiation of ER stress-mediated apoptosis.

4.3.3. The antiproliferative activity of AEA was not mediated by cannabinoid receptors

The cytotoxic effects of AEA are regulated by cannabinoid receptor-dependent and -independent pathways (Van Dross R. et al., 2013). CB1 and CB2 receptors are G_i-coupled receptors that inactivate adenylate cyclase thereby reducing cAMP levels in the presence of cannabinoid receptor agonists. JWF2 cells were pretreated with selective CB1R (AM251) and CB2R (AM630) antagonists to identify concentrations that prevented the agonist-mediated reduction in cAMP. In AEA-treated cells, the reduction in cAMP was inhibited by 0.001-0.1 μM

AM251 and AM630. In contrast, this trend was reversed by 1 μ M of AM251 and AM630 (Figure 4.4A). Therefore, cells were treated with 0.1 μ M of the receptor antagonists in our studies. To determine whether AEA-induced apoptosis required cannabinoid receptor activation, JWF2 cells were pre-incubated with AM251 or AM630 and then treated with AEA. Antagonism of the CB1 or CB2 receptor did not inhibit AEA-induced caspase 3 cleavage (Figure 4.4B & 4.4C). The effect of the cannabinoid receptors on the antiproliferative activity of AEA was also examined. AEA-mediated cell death occurred despite the presence of the CB1 and CB2 receptor antagonists. Hence, AEA-induced apoptosis and cell death occur through a receptor-independent mechanism.

4.3.4. AEA-induced oxidative stress and ER stress were not mediated by the cannabinoid receptors

Oxidative- and ER-stress are necessary for the initiation of apoptosis by AEA (Figure 3.5B and Figure 4.3B). Therefore, we examined whether AEA-induced oxidative- and ER-stress were regulated by the cannabinoid receptors. As we observed previously, AEA increased oxidative stress in JWF2 cells. Pretreatment of the cells with AM251 or AM630 did not block the production of oxidative stress caused by AEA (Figure 4.5A & 4.5B). Similarly, the increase in CHOP10 expression mediated by AEA was not prevented by cannabinoid receptor inactivation (Figure 4.5C & 4.5D). Collectively, these results imply that AEA-induced ER stress-mediated apoptosis is a process that does not require cannabinoid receptor activity.

4.4. FIGURES

Figure 4.1: AEA induced oxidative stress and ER stress in NMSCs

(A) Cell viability was measured by MTS assay in JWF2 cells treated with the indicated concentration of AEA for 8 hours. (B) JWF2 cells were treated with AEA (20 μ M) or vehicle (ETOH) for 4 and 6 hours. CHOP10 expression and cleaved caspase3 (Cl- Casp3) were analyzed by Western blot analysis. GAPDH expression was measured as loading control. Band intensities were quantified using ImageJ software. Fold induction was determined by comparing the band intensities of samples to vehicle-treated cells after normalizing GAPDH levels. (C-D) JWF2 cells were treated with vehicle (ETOH), H₂O₂ (50 μ M), AEA (10 and 20 μ M) and 15-deoxyPGJ₂ (5 μ M, positive control) for 1 hour. (C) Oxidative stress was measured by flow cytometry using CM-H₂DCFDA reagent. Fluorescence was measured as a percent increase from control untreated cells. (D) The intracellular level of total glutathione was measured using DTNB reagent. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* indicates the statistically significant difference between the samples and vehicle treated cells, P<0.05). (E-F) JWF2 cells were pretreated with CM-H₂DCFDA and then treated with vehicle (ETOH) or 20 μ M AEA for 1 hour. DCF fluorescence (E, upper panel) and phosphorylated eIF2 α (F, upper panel) were measured using flow cytometry and analyzed with a BD FACSCanto II flowcytometer with BD FACSdiva software (BD Biosciences). Red peaks correspond to AEA treated cells and black peaks correspond to vehicle treated cells. Mean fluorescence intensities in AEA and vehicle treated cells were represented as mean \pm SEM (E-F, lower panels). Data were analyzed using Student's t-test (* indicates the statistically significant difference between AEA and vehicle treated cells, P<0.05).

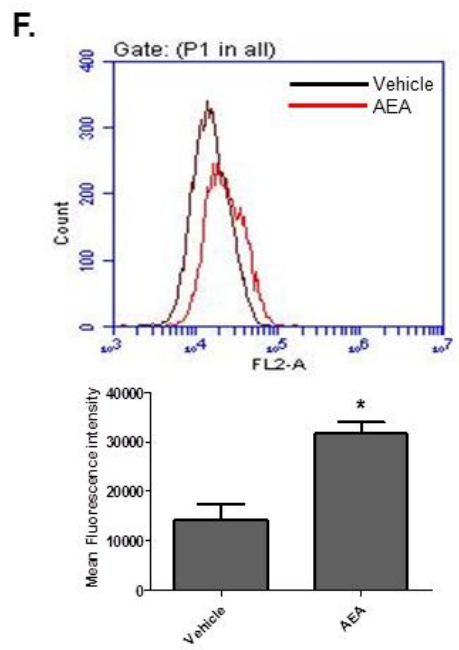
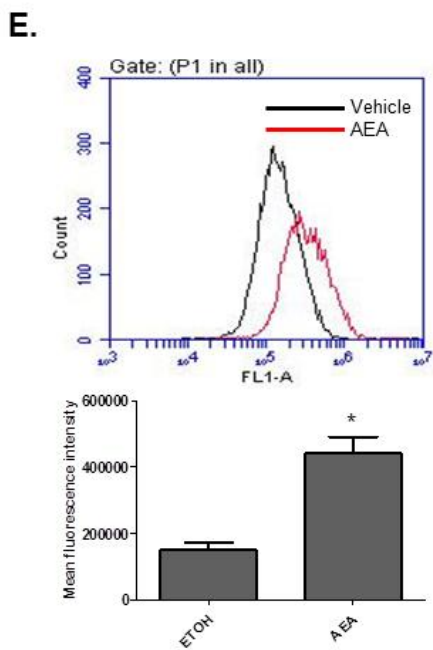
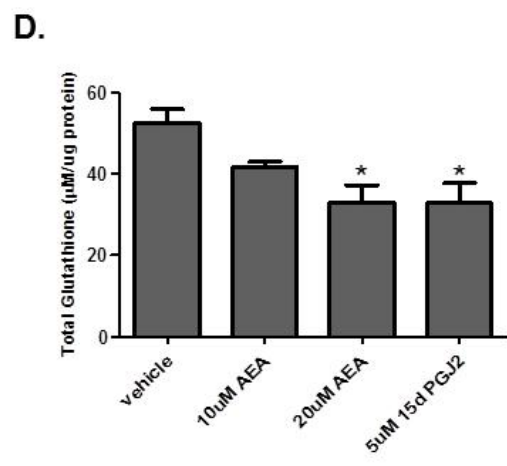
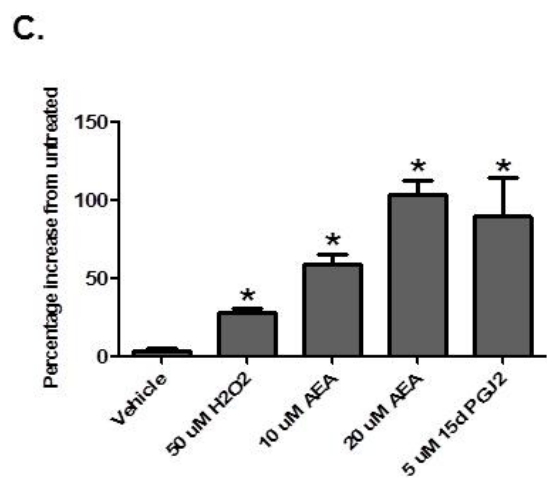
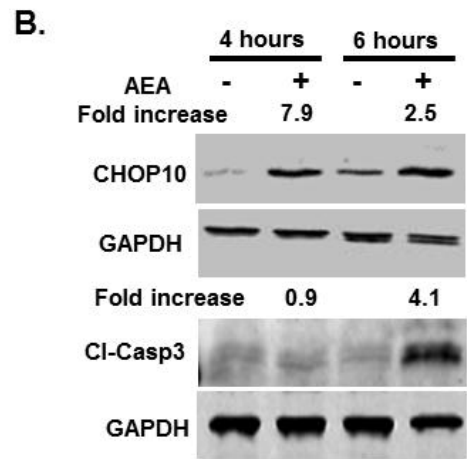
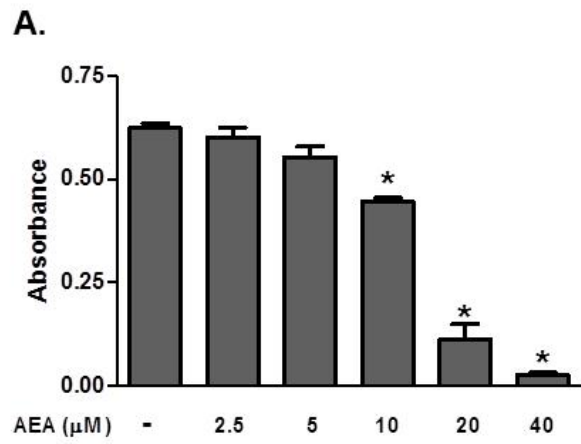


Figure 4.2: Antioxidants inhibited AEA-induced oxidative stress and cell death

(A-B) NAC inhibited AEA-induced oxidative stress and GSH reduction. JWF2 cells were pretreated with NAC (25 or 50 mM) for 2 hours and then treated with AEA (20 μ M) for 1 hour. (A) Oxidative stress was evaluated by flow cytometry using CM-H₂DCFDA reagent. DCF fluorescence was measured as a percent increase from control untreated cells. (B) Intracellular total glutathione level was measured using DTNB reagent. (C-D) Trolox inhibited AEA-induced oxidative stress but not GSH reduction. JWF2 cells were pretreated with Trolox (0.25 or 0.5 mM) for 1 hour and then treated with AEA (20 μ M) for 1 hour. (C) Oxidative stress was evaluated by flow cytometry using CM-H₂DCFDA reagent. DCF fluorescence was measured as a percent increase from control untreated cells. (D) Intracellular total glutathione level was measured using DTNB reagent. (E-F) NAC and Trolox inhibited the antiproliferative effect of AEA. JWF2 cells were pretreated with NAC (25 or 50 mM) for 2 hours (E) or Trolox (0.25 or 0.5 mM) for 1 hour (F) and then treated with AEA (20 μ M) for 8 hours. Cell viability was measured using MTS assay. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* indicates the statistically significant difference between the samples and vehicle-treated cells. # indicates the statistically significant difference between the samples and AEA-treated cells. P<0.05).

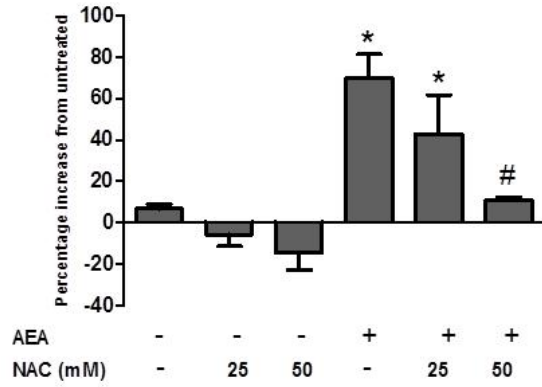
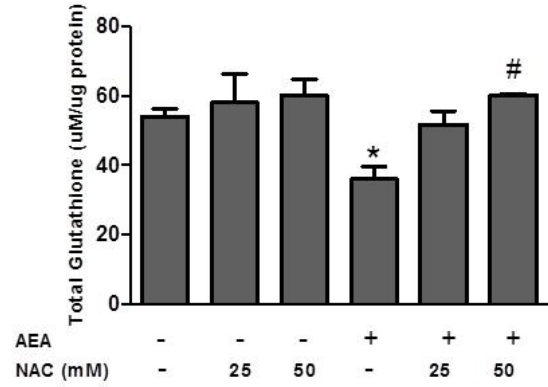
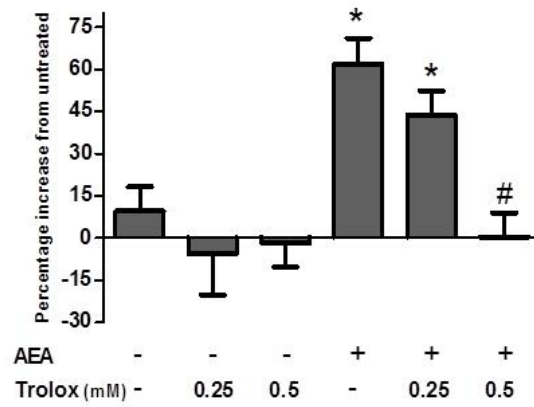
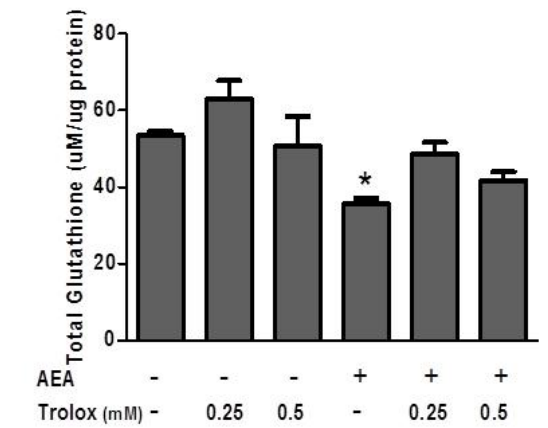
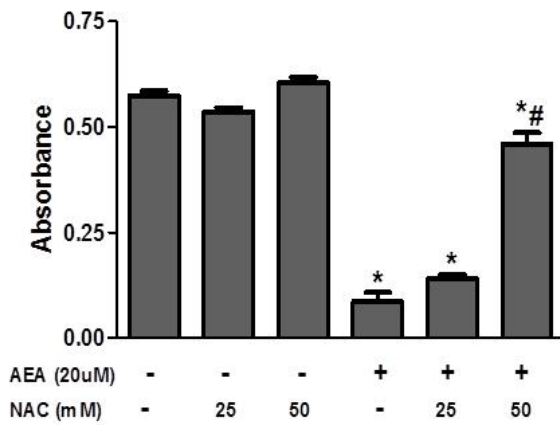
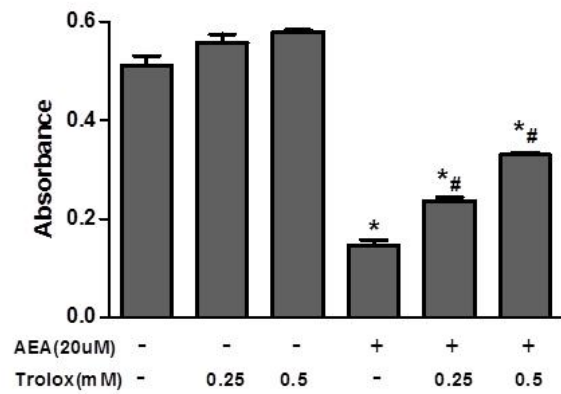
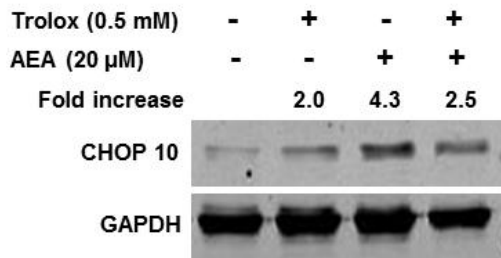
A.**B.****C.****D.****E.****F.**

Figure 4.3: Oxidative stress is required for the initiation of ER stress-apoptosis by AEA

JWF2 cells were pretreated with 0.5mM Trolox for 1 hour and then treated with AEA. (A) CHOP 10 expression (4 hours), (B) Cleaved Caspase 3 (Cl-Casp3) (6 hours) and GAPDH expressions (loading control) were analyzed by Western blot analysis. Band intensities were quantified using ImageJ software. Fold induction in protein expression was determined by comparing the band intensities of samples to vehicle-treated cells after normalizing GAPDH levels.

A.



B.

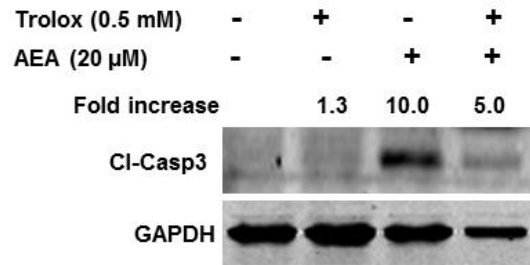
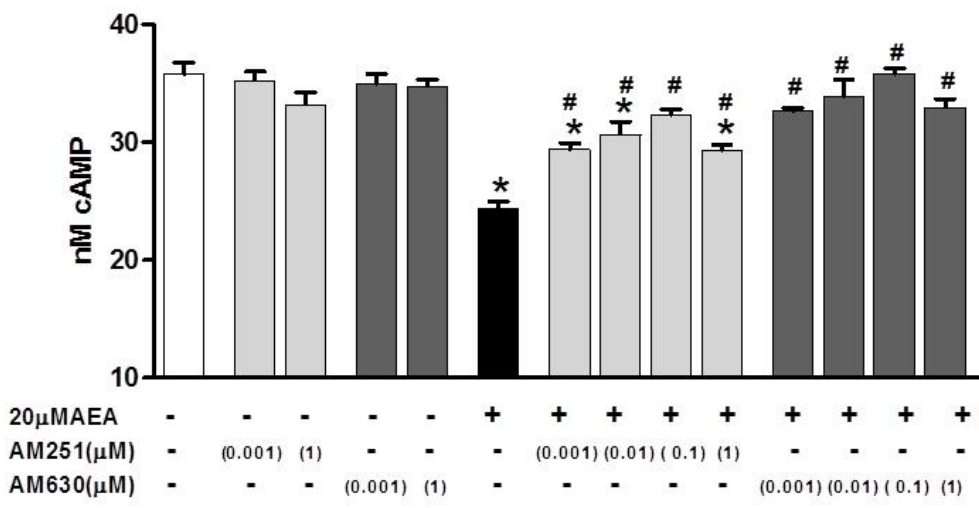


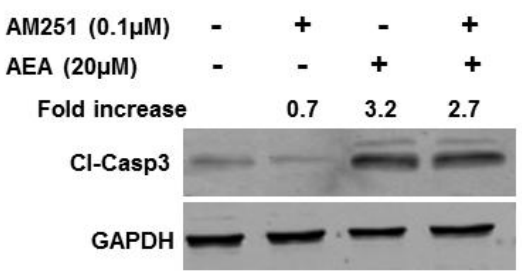
Figure 4.4: The apoptotic effect of AEA on NMSC was not mediated by cannabinoid receptors

(A) Selective CB1 (AM251) and CB2 (AM630) receptors antagonists inhibited the effect of AEA on the reduction of cAMP. JWF2 cells were pre-treated with the indicated concentrations of AM251 and AM630 for one hour and then treated with 20 μ M AEA for 15 minutes. cAMP was measured using the cAMP Glo ELISA kit. (B-D) AEA-induced apoptotic cell death was not mediated by CB1 or CB2 receptors. JWF2 cells were pretreated with 0.1 mM AM251 or AM630 and then treated with vehicle (ETOH), or AEA (20 μ M) for 4 and 8 hours. (B, C) Caspase 3 cleavage and GAPDH (loading control) were analyzed by Western blot. Fold induction in protein expression was determined by comparing the band intensities of samples to vehicle-treated cells. (D) Cell viability was measured after 8 hours by MTS assay. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* indicates the statistically significant difference between the samples and vehicle treated cells and # indicates the statistically significant difference between the samples and AEA- treated cells, P<0.05).

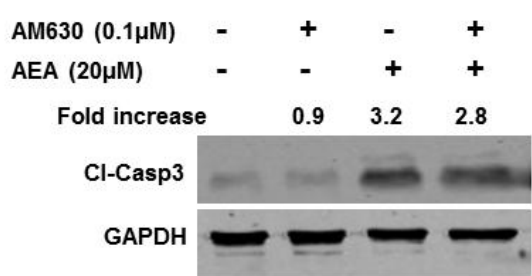
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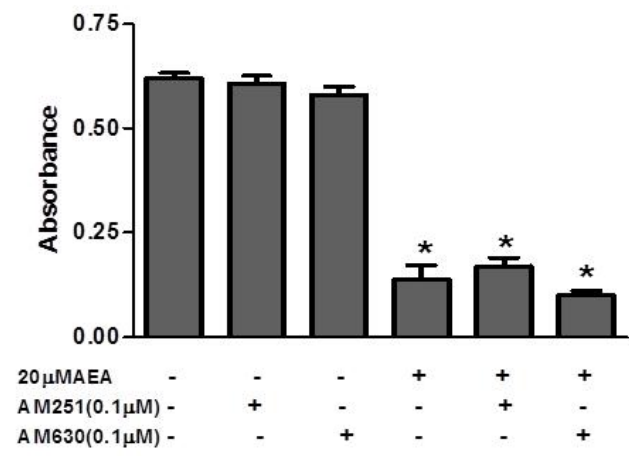
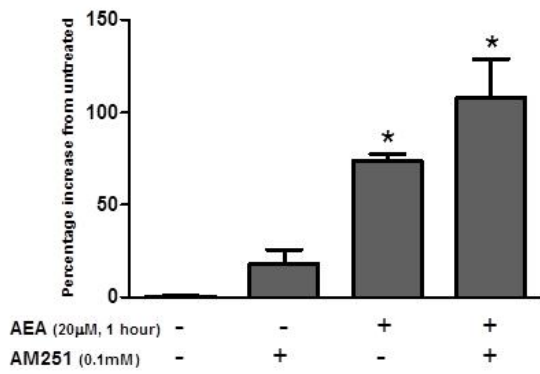


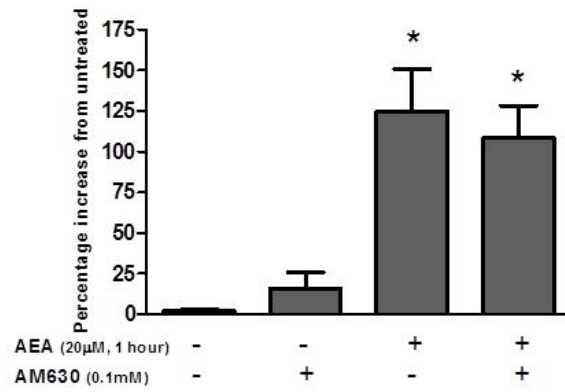
Figure 4.5: Oxidative and endoplasmic reticulum stress induced by AEA was not mediated by cannabinoid receptors

(A, B) AEA-induced oxidative stress was not mediated by cannabinoid receptors CB1/2. JWF2 cells were pre-treated with 0.1 mM AM251 (A) or 0.1 mM AM630 (B) for one hour and then treated with 20 μ M AEA for 1 hour. Oxidative stress was measured as a percent increase in DCF fluorescence from untreated cells. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean \pm SEM (* indicates the statistically significant difference between the samples and vehicle treated cells and # indicates the statistically significant difference between the samples and AEA- treated cells, $P < 0.05$). (C, D) AEA-induced ER stress was not mediated by CB1/2 receptors. JWF2 cells were pretreated with 0.1 mM AM251 or AM630 and then treated with vehicle (ETOH), or AEA (20 μ M) for 4 hours. CHOP 10 and GAPDH expression (loading control) were analyzed by Western blot. Fold induction in protein expression was determined by comparing the band intensities of samples to vehicle-treated cells.

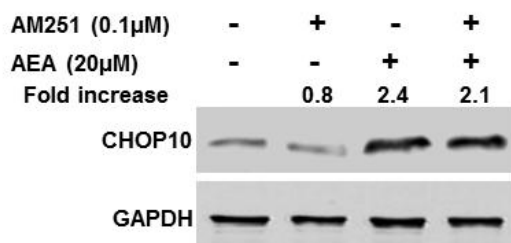
A.



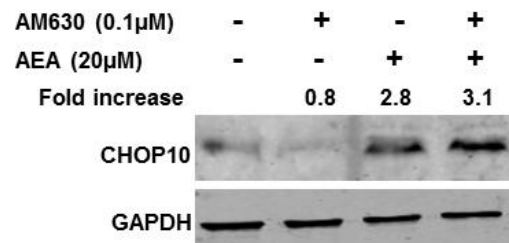
B.



C.



D.



4.6. DISCUSSION

Non melanoma skin cancer (NMSC) is the most common cancer in the United States. NMSC is associated with substantial annual health care costs, which are expected to rise dramatically due to excessive sun and UV-light exposure (Diepgen and Mahler, 2002; Guy and Ekwueme, 2011). Accordingly, novel treatments are required to address this issue. The endocannabinoid, AEA, is a potential therapeutic for NMSC. In chapter three, we showed that AEA selectively induced ER stress-mediated apoptosis in NMSC cells that overexpress COX-2 (Soliman et al., 2014). Specifically, we demonstrated that the cytotoxic effects of AEA were most likely produced by the novel prostaglandin, 15d-PGJ₂-EA, which was synthesized as a consequence of the metabolism of AEA by COX-2. In this Chapter, we demonstrate that AEA-induced ER stress-apoptosis was also regulated by oxidative stress that was partially mediated by a reduction in total GSH levels. In contrast, cannabinoid receptor activation was not required for oxidative stress, ER stress or apoptosis that was caused by AEA. Thus, cell death in NMSC cells is likely regulated by the conversion of AEA to 15d-PGJ₂-EA, which diminishes intracellular glutathione levels leading to oxidative stress, ER stress and ultimately apoptosis (Figure 4.6).

Oxidative stress plays an important role in the promotion and prevention of carcinogenesis. Relatively high levels of reactive molecules are required to activate proliferative and pro-survival signal transduction pathways while; excessive oxidative stress causes sufficient damage to cellular DNA, proteins and lipids to activate cell death pathways (Glasauer and Chandel, 2014). Thus, a therapeutic strategy that has been employed to elicit tumor cell death is to utilize agents that induce excessive oxidative stress. Several clinically available anticancer agents including doxorubicin and bleomycin induce tumor cell death by initiating lethal oxidative stress (Burger et al., 1981; Thorn et al., 2011). However, additional studies are needed

to fully characterize the oxidative properties of AEA and the role of oxidative stress in AEA-induced tumor cell death. It has been reported that AEA-induced cell death was prevented by the antioxidants, NAC, GSH, and ebselen in head and neck squamous cell carcinoma cells (Park et al., 2014). In addition, we showed previously that inhibiting the generation of oxidative stress with NAC blocked AEA-mediated apoptosis in tumorigenic keratinocytes. In this study, we report that oxidative stress was also required for ER stress-mediated apoptosis (Figure 4.3). In addition, our data suggested that AEA-induced oxidative stress was caused by a reduction in the intracellular glutathione levels. In most cells, glutathione is the primary antioxidant that neutralizes reactive oxygen/nitrogen species and electrophilic molecules (Ribas et al., 2014). Diminished glutathione levels weaken the cell's defense against reactive molecules leading to excessive oxidative stress and cell death. Consistent with these observations, Siegmund et al. demonstrated that the susceptibility of hepatic stellate cells to AEA-induced cell death correlated with the cellular GSH content with high levels of GSH conferring resistance and low levels promoting sensitivity to cell death (Siegmund et al., 2005; Siegmund et al., 2006). Interestingly, a different study showed that GSH also regulated J-series prostaglandin-induced cell death (Kondo et al., 2001). 15d-PGJ₂ is a cyclopentenone prostaglandin that possesses a highly reactive α , β -unsaturated carbonyl group in its cyclopentenone ring, which can interact via a Michael addition reaction with nucleophiles including free sulfhydryls of cysteine residues in cellular proteins. Covalent binding of J-series PGs with GSH and other proteins that regulate cellular redox status inactivate these antioxidant enzymes thereby increasing ROS (Uchida and Shibata, 2008). Since AEA is metabolized to 15d-PGJ₂-EA, which possesses the conserved α , β -unsaturated carbonyl group, it is highly probable that 15d-PGJ₂-EA also binds to and reduces GSH levels in AEA-treated cells. Hence, our supposition that a reduction in intracellular

glutathione is involved in the initiation of lethal oxidative stress in AEA-treated cells is consistent with our presented data and the established prooxidant activity of the J-series prostaglandins.

Although our data indicate that the activity of AEA/15d-PGJ₂-EA appears to be controlled primarily by GSH, AEA-mediated ER stress-apoptosis may also be regulated by oxidative stress. NAC is a thiol antioxidant which serves as a prodrug to L-cysteine, the limiting agent in GSH synthesis (Zafarullah et al., 2003). Trolox, on the other hand, is an antioxidant which acts primarily by scavenging the free radicals (Mazor et al., 2006). In Figure (4.2) we demonstrated that Trolox and NAC inhibited AEA-induced oxidative stress and cell death however, cell treatment with NAC but not Trolox produced a complete restoration of GSH levels. This suggests that since the cells were rescued from cell death by an antioxidant that primarily scavenges free radicals that ROS may also be a key initiator of oxidative stress. This idea is supported by Kondo et al who demonstrated that 15d-PGJ₂ increased the generation of the highly reactive molecules, 4-hydroxy-2-nonenal (HNE) and acrolein, and also decreased the transient mitochondrial membrane potential (Kondo et al., 2001). Thus, cytotoxic oxidative stress generated by AEA is likely caused by multiple mechanisms including the modulation antioxidant enzyme levels and the production of reactive cellular molecules.

Reactive oxygen species (ROS) have emerged as important regulators of physiological ER activity as well as initiators of ER stress. Although the molecular events linking ROS to components of the ER stress pathway are still obscure, evidence connecting these distinct processes is increasing. For example, in several diseased conditions, ER stress and increased ROS production occur concurrently (Malhotra and Kaufman, 2007). In addition, Verfaillie et al reported that PERK, which is a key mediator of ER stress signaling, is located at the ER-

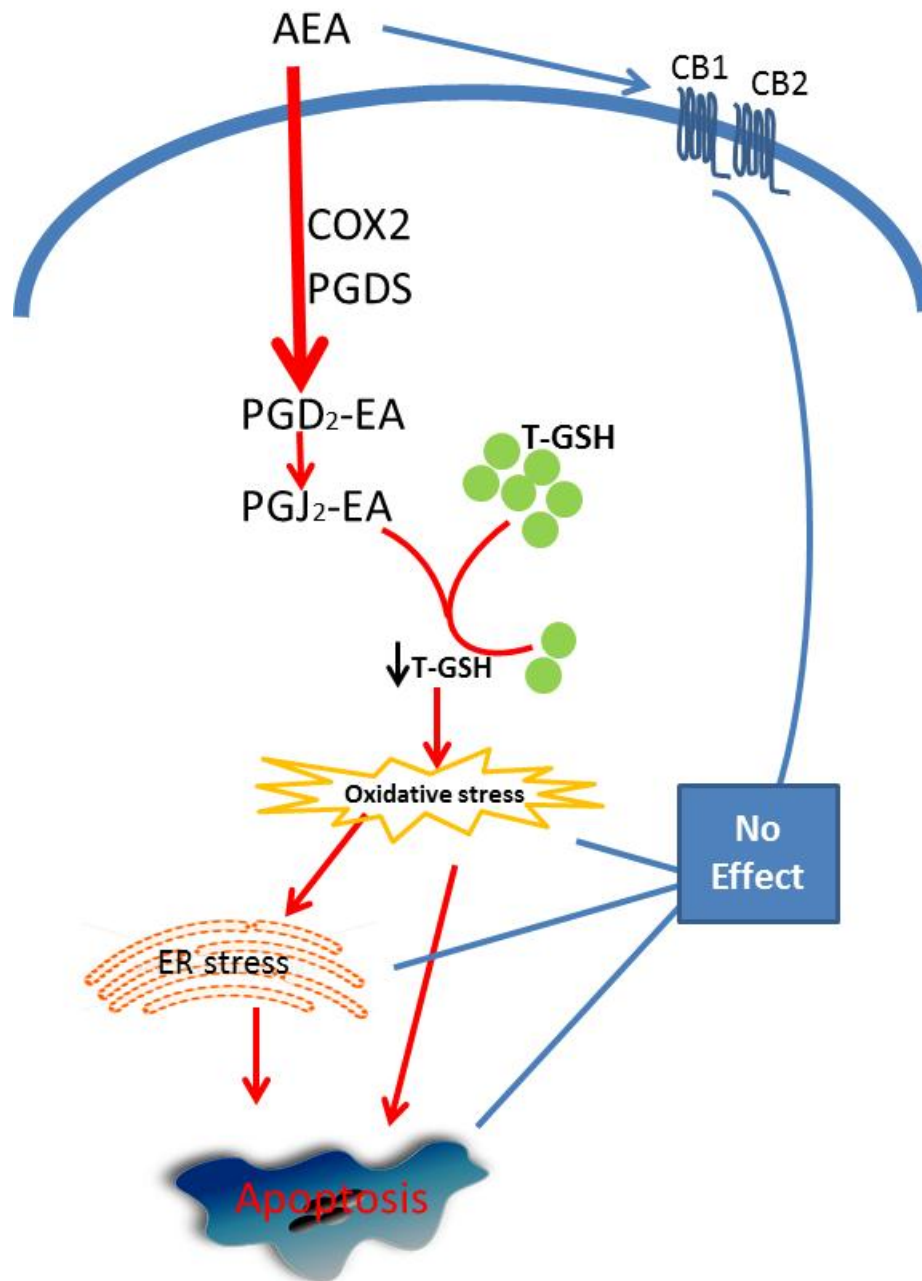
mitochondria contact sites and is required to induce apoptosis after oxidative stress-dependent ER stress (Verfaillie et al., 2012). Consistent with these findings our data show that the antioxidant Trolox inhibited AEA-induced CHOP 10 expression and caspase 3 cleavage (Figure 4.3) indicating that ROS production is important for ER stress-apoptosis. Similarly, Su and associates showed that ROS was required for ER stress and apoptosis in cells treated with 15d-PGJ₂ (Su et al., 2008). Collectively, these findings suggest that the induction of oxidative stress by AEA likely leads to the activation ER stress and the initiation of apoptosis.

Several *in vivo* and *in vitro* studies have reported that cannabinoid-induced cell death occurs via CB1/2 receptor-dependent and -independent pathways (Van Dross R. et al., 2013). For example, CB1 receptors were reported to mediate ER stress-apoptosis induced by AEA in neuroblastoma cells (Pasquariello et al., 2009) and by tetrahydrocannabinol (THC) in glioma cells (Salazar et al., 2009). Furthermore, in hyperplastic cholangiocytes, AEA-induced apoptosis required CB2 receptor-dependent activation of thioredoxin 1/ redox factor 1 and AP1 (DeMorrow et al., 2007). In contrast, CB1- and CB2-independent cell death has also been observed in several reports. The use of CB1 and CB2 receptor antagonists did not inhibit AEA-induced cell death in human colorectal cancer cells (Gustafsson et al., 2009; Patsos et al., 2010). In addition, AEA-induced cell death was initiated independent of CB1, CB2 and the endocannabinoid-sensitive ion channel, TRPV1 in tumorigenic keratinocytes (Kuc et al., 2012). In the current study, we observed that pharmacological antagonism of the CB1 and CB2 receptors did not inhibit AEA-induced oxidative stress, ER stress or apoptosis in NMSC cells (Figure 4.4-4.5). Since we also demonstrated that cannabinoid receptor levels were not modulated by cell exposure to AEA (Kuc et al., 2012), it is unlikely that the inability of CB1/2 antagonist to inhibit the cytotoxicity of AEA was due to the down regulation of these receptors.

Collectively, these findings show that oxidative stress is required for AEA-induced ER stress and apoptosis. Furthermore, our findings indicate that although AEA is a cannabinoid receptor agonist, the cytotoxicity of this agent is not reliant on these receptors. Hence, one can reasonably conclude that AEA is metabolized to 15d-PGJ₂-EA, which initiates oxidative stress and triggers ER stress-mediated apoptosis.

Figure 4.6: Proposed pathway of AEA-induced apoptosis in NMSC cells

We propose that in tumor cells which overexpress COX-2, AEA is metabolized by COX-2 followed by prostaglandin D synthase (PGDS) to ethanolamide conjugated J-series prostaglandins that decrease total intracellular Glutathione (GSH) thereby increasing the sensitivity of the cells to oxidative stress. The induction of oxidative stress then leads to ER stress, which then causes apoptosis. The effects of AEA on oxidative stress, ER stress, and apoptosis are not mediated by CB1 or CB2 receptors.



CHAPTER FIVE: GENERAL DISCUSSION AND SUMMARY

The main goal of the present study was to develop a novel therapeutic for NMSC that is selectively toxic to cancer cells but has minimal toxicity in the normal surrounding cells. To achieve this goal, we took advantage of the fact that COX-2 is overexpressed in tumor, but not in non-tumor, cells (Figure 3.1B). COX-2 metabolizes AEA to PGD₂-EA, PGE₂-EA and PGF_{2 α} -EA (Kozak et al., 2002a). Our data provided the first evidence that AEA is also converted to the cytotoxic J- series prostaglandin-ethanolamide, 15d-PGJ₂-EA (Figure 3.6). Therefore, we hypothesized that AEA would be metabolized to 15dPGJ₂-EA in tumor cells that overexpress COX-2 but not normal keratinocytes, which contain low endogenous levels of COX-2. Indeed, our data showed that AEA preferentially inhibited the survival of tumorigenic JWF2, but not non-tumorigenic HaCaT keratinocytes (Figure 3.1A). Furthermore, we observed that the antiproliferative effect of AEA correlated with the magnitude of COX-2 expression (Figure 3.2) suggesting that this enzyme plays a crucial role in AEA cytotoxicity. Hence, the main objectives of the present study were to determine whether the cytotoxicity of AEA was mediated by 15d-PGJ₂-EA and to uncover molecular mechanisms by which AEA-induced apoptosis occurs.

In the first part of this study, we confirmed our previous finding that AEA induced NMSC cell apoptosis (Figure 3.4). We also determined that AEA caused ER stress as indicated by the activation of PERK, ATF6, and IRE1 signaling pathways (Figure 3.3). In addition, the ER stress-associated pro-apoptotic proteins CHOP10 and caspase 12 were activated prior to the initiation of apoptosis (Figure 3.4). Moreover, ER stress was necessary for the induction of apoptosis by AEA (Figure 3.5). The role of COX-2 in AEA-induced ER stress-apoptosis was also examined and it was determined that COX-2 was required for ER stress in the presence of AEA (Figure 3.7 and 3.8). Collectively, these data suggested that COX-2 regulated AEA-

mediated ER stress-apoptosis by converting AEA to 15d-PGJ₂-EA. In support of this contention, several studies show that AEA and other cannabinoids induce cytotoxic ER stress in cancer cells (Carracedo et al., 2006;Pasquariello et al., 2009). In addition, J-series prostaglandins, which are derived from AA initiate apoptosis via the ER stress pathway (Su et al., 2008). Thus, AEA-induced ER stress-apoptosis is likely mediated by 15d-PGJ₂-EA.

To establish the requirement for 15d-PGJ₂-EA in AEA-induced cell death, the synthesis of D- and J-series PGs was blocked using a selective inhibitor of PGDS. Blockade of PGDS activity prevented AEA-induced ER stress and apoptosis (Figure 3.9E). Moreover, exogenous PGD₂-EA was converted to J-series prostamides and also induced ER stress-apoptosis (Figure 3.10). Other reports have shown that COX-2 was necessary for AEA-induced tumor cell death (Ramer et al., 2001;Patsos et al., 2010). However, our data provided the first evidence that AEA-induced apoptosis was due to the production of 15d-PGJ₂-EA.

In the second part of this study we sought to determine: 1) the role of oxidative stress in AEA-induced ER stress-apoptosis, and 2) the role of the cannabinoid receptors in oxidative stress, ER stress and apoptosis. Our data indicated that a reduction in intracellular GSH and an increase in intracellular ROS were the most likely causes of AEA-induced oxidative stress (Figure 4.1). We also determined that antioxidants could prevent AEA-mediated oxidative stress and apoptosis. Furthermore, an antioxidant blocked AEA-initiated ER stress suggesting that oxidative stress was required for AEA-induced ER stress-apoptosis. Consistent with these results, Gustafson et al showed that AEA-induced apoptosis occurred in an oxidative stress-dependent fashion in colorectal carcinoma cells (Gustafsson et al., 2009). In addition, ROS production induced by 15d-PGJ₂ was caused by the interaction of 15d-PGJ₂ with GSH (Kondo et al., 2001;Uchida and Shibata, 2008). Furthermore, 15d-PGJ₂-induced ROS was shown to

regulate ER stress and apoptosis (Su et al., 2008). Thus, the generation of oxidative stress is essential for the induction of ER stress-mediated apoptosis in tumor cells that are treated with AEA.

The data presented in this study also firmly establish that AEA-induced apoptosis does not require the activity of the cannabinoid receptors. Specifically, we demonstrated that selective antagonism of the CB1 or CB2 receptors did not inhibit AEA-induced oxidative stress, ER stress, or apoptosis (Figure 4.4 and 4.5). In agreement of these observations, CB1 and CB2 receptor antagonists did not inhibit AEA-induced cell death in human colorectal cancer cells (Gustafsson et al., 2009; Patsos et al., 2010). Thus, the present findings indicate that although AEA is a cannabinoid receptor agonist, the cytotoxicity of this agent does not require these receptors.

In summary, the data presented in this report show that AEA is cytotoxic to cells that overexpress COX-2. We have identified novel, 15d-PGJ₂-EA, as a likely regulator of AEA-mediated tumor cell apoptosis, which our data suggests occurs via the initiation of oxidative stress and ER stress in a cannabinoid receptor-independent manner. Since non-tumorigenic keratinocytes possess low endogenous levels of COX-2, 15d-PGJ₂-EA is not synthesized and minimal toxicity occurs in these cells. As such, AEA and AEA derivatives may be ideal agents for topical treatment of NMSC and other cancers that overexpress COX-2.

FUTURE DIRECTIONS

The widely used pharmacological treatment for NMSC is 5-fluorouracil (5-FU). 5-FU induces cell cycle arrest and apoptosis by blocking DNA synthesis in rapidly dividing cancer cells. Since normal healthy skin cells also divide rapidly, they are subject to 5-FU cytotoxicity. Our *in vitro* findings suggest that AEA is preferentially cytotoxic to tumorigenic compared to non-tumorigenic keratinocytes. We also demonstrated that the tumor cell toxicity of AEA is attributed to COX-2 and the production of the cytotoxic metabolite, 15dPGJ₂-EA. Therefore, future investigations will assess the antitumor activity of AEA *in vivo*. In these studies, the xenograft tumor model will be used to evaluate the anticancer activity of AEA. COX-2 overexpressing cells will be implanted subcutaneously in the flank region of mice and AEA will be injected into the tumor to examine its effect on tumor growth. Also, the synthesis of 15dPGJ₂-EA as well as the induction of oxidative stress, ER stress and apoptosis will be evaluated in the tumors. Additional studies will be conducted to examine the safety and pharmacokinetic activity of AEA when topically applied to normal mouse skin. Such an investigation will unveil the chemotherapeutic properties of AEA against NMSC.

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