Omega-3 polyunsaturated fatty acids and their impact upon the biosynthesis of endocannabinoids and $N$-acylethanolamines in human skin cells in the presence and absence of ultraviolet radiation

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<th>Full Form</th>
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<td>1-AG</td>
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<td>Diacylglycerol lipase</td>
</tr>
<tr>
<td>DGLA</td>
<td>Dihomo γ linolenic acid</td>
</tr>
<tr>
<td>DGLEA</td>
<td>Dihomo γ linolenoyl ethanolamide</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DHEA</td>
<td>Docosahexaenoyl ethanolamide</td>
</tr>
<tr>
<td>DHET</td>
<td>Dihydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DT</td>
<td>Delayed tanning</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Culture</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemilluminescent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EPEA</td>
<td>Eicosapentaenoyl ethanolamide</td>
</tr>
<tr>
<td>ES+</td>
<td>Positive ionization mode</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLA</td>
<td>γ Linolenic acid</td>
</tr>
<tr>
<td>GP-AEA</td>
<td>Glycerophospho-arachidonoyl ethanolamide</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>HaCaT</td>
<td>Human adult, calcium, temperature</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxy eicosatetraenec acid</td>
</tr>
<tr>
<td>HODEs</td>
<td>Hydroxyoctadecadienoic acids</td>
</tr>
<tr>
<td>HPEPE</td>
<td>Hydroperoxy eicosapentaenoic acid</td>
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21
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>HPETE</td>
<td>Hydroperoxy eicosatetraenoic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxide</td>
</tr>
<tr>
<td>iPLA2</td>
<td>Independent cytosolic Ca(^{2+}) phospholipase A2</td>
</tr>
<tr>
<td>LA</td>
<td>Linolenic acid</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LEA</td>
<td>Linoleoylethanolamide</td>
</tr>
<tr>
<td>LOXs</td>
<td>Lipoxigenases</td>
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<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>LTA4</td>
<td>Leukotriene A4</td>
</tr>
<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>LXA4</td>
<td>Lipoxin A4</td>
</tr>
<tr>
<td>LXB4</td>
<td>Lipoxin B4</td>
</tr>
<tr>
<td>Lyso-N-PE</td>
<td>Lyso-N-arachidonoyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>Lyso-PA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>Lyso-PLD</td>
<td>Lysophospholipase D</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MAR</td>
<td>Maresins</td>
</tr>
<tr>
<td>MED</td>
<td>Minimal erythema dose</td>
</tr>
<tr>
<td>MEME</td>
<td>Minimum essential medium eagle</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>Omega-3 polyunsaturated fatty acid</td>
</tr>
<tr>
<td>n-6 PUFA</td>
<td>Omega-6 polyunsaturated fatty acid</td>
</tr>
<tr>
<td>N(_2)</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NAAA</td>
<td>N-acylethanolamine hydrolysing acid amidase</td>
</tr>
<tr>
<td>NADA</td>
<td>N-arachidonoyl-dopamine</td>
</tr>
<tr>
<td>NAEs</td>
<td>N-acyl ethanolamines</td>
</tr>
<tr>
<td>NAPE</td>
<td>N-arachidonoyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acyl phosphatidylethanolamine phospholipase D</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acyltransferase</td>
</tr>
<tr>
<td>NHAK</td>
<td>Normal human adult keratinocytes</td>
</tr>
<tr>
<td>NHEKs</td>
<td>Cultured normal human epidermal keratinocytes</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>OEA</td>
<td>Oleoylethanolamide</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDs</td>
<td>Protectins</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEA</td>
<td>N-palmitoylethanolamide</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGDS</td>
<td>Prostaglandin D synthases</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E(_2)</td>
</tr>
<tr>
<td>PGES</td>
<td>Prostaglandin E synthase</td>
</tr>
<tr>
<td>PGF5</td>
<td>Prostaglandin F synthase</td>
</tr>
<tr>
<td>PGG2</td>
<td>Prostaglandin G(_2)</td>
</tr>
<tr>
<td>PGH2</td>
<td>Prostaglandin H(_2)</td>
</tr>
<tr>
<td>PIC</td>
<td>Protease inhibitor cocktail</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PLA(_2)</td>
<td>Phospholipase A(_2)</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin streptomycin</td>
</tr>
<tr>
<td>PtdCho</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase, non-receptor type 22</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RvD</td>
<td>D-series resolvins</td>
</tr>
<tr>
<td>RvE</td>
<td>E-series resolvins</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SED</td>
<td>Standard erythemal dose</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>sPLA2</td>
<td>Secretary phospholipases A2</td>
</tr>
<tr>
<td>STEA</td>
<td>N-stearoylethanolamine</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylenediamine</td>
</tr>
<tr>
<td>THC</td>
<td>Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential channel type V1</td>
</tr>
<tr>
<td>TXAS</td>
<td>Thromboxane A synthase</td>
</tr>
<tr>
<td>UCA</td>
<td>Urocanic acid</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet radiation</td>
</tr>
<tr>
<td>Abhd 4</td>
<td>αβ-hydrolase 4</td>
</tr>
</tbody>
</table>
Abdalla F. Mohammed Almaedani
Doctor of Science (DSc)

Omega-3 polyunsaturated fatty acids and their impact upon the biosynthesis of endocannabinoids and N-acylethanolamines in human skin cells in the presence and absence of ultraviolet radiation.
March 2015

Abstract

Endocannabinoids are endogenous lipid mediators involved in various biological processes, and have immunomodulatory and anti-inflammatory activities. Anandamide (arachidonoyl ethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG) are the main representatives of this group. The endocannabinoid receptors CB1 and CB2 with AEA have been found in human HaCaT keratinocytes and fibroblasts, but the metabolic pathway leading to endocannabinoid production in the skin has not been fully elucidated. This study aimed to investigate the profile of endocannabinoids and their main metabolizing enzymes in human skin cells and assess whether omega-3 polyunsaturated fatty acids (n-3 PUFA) altered these profiles. In addition, an investigation was carried out to check whether UV radiation could stimulate the production of endocannabinoids and N-acylethanolamines (NAE) in human skin cells. For this purpose HaCaT keratinocytes and 46RB.1N fibroblast cells were treated with 10 and 50µM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or oleic acid (OA) in the presence or absence of UVR (15mJ/cm2). Data suggest that n-3 PUFA may both directly (by up-regulating NAPE-PLD levels) and indirectly (by decreasing FAAH levels) increased endocannabinoid and NAE levels in HaCaT keratinocytes and 46BR.IN fibroblasts. DHA treatment significantly decreased COX-2 expression in the absence of UVR and inhibited UVR-induced COX-2 overexpression in 46BR.IN fibroblasts. In contrast, DHA appeared to induce COX-2 up-regulation in the absence of UVR and did not prevent UVR induced COX-2 up-regulation in HaCaT keratinocytes. EPA appeared to induce COX-2 down-regulation in the absence of UVR and did not prevent UVR induced COX-2 up-regulation in both HaCaT keratinocytes and 46BR.IN fibroblasts. UVR did not have any significant effect on endocannabinoid and NAE biosynthesis. However, UVR induced endocannabinoid production in some experiments of this study. A clinical study was carried on 16 volunteers from two different ethnic groups and two different skin types. The purpose was to assess the effect of UVR on the serum endocannabinoids and NAE, therefore, the volunteers were subjected to multiple doses (1.3, SED/6 min) of UVR for 6 weeks. Data showed that UVR did not have major effect on human serum NAE in both skin phototypes II and V but increased 2-AG in human serum in both skin types but the more pronounced effect was evident in skin phototypes V rather than in skin phototypes II. Human serum docosahexaenoylethanolamide levels were found to be higher in White Caucasians group (skin phototypes II). Based on these it can be concluded that n-3 PUFA and UVR alter the endocannabinoids and NAE profile in HaCaT keratinocytes and 46BR.IN fibroblasts. In addition, results of the clinical study indicated that UVR has no major effects on serum endocannabinoids or NAE therefore, further studies are required to address this question in vivo.
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I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Finally I cannot end without conveying a deepest thanks my whole family especially my wife Najah for her patience, support and for helping me in any possible way to accomplish my degree.
This thesis is dedicated to my brother

Ibrahim for his endless support,

generosity and encouragement
CHAPTER 1: Introduction

1.1. Introduction to human skin biology

The skin is the largest organ of the body, it is forms the physical barrier that protect the body from environmental stress and regulates water inward and outward (Hunter, 2003). Skin also has a protection role against microorganisms, chemical agents and ultraviolet radiation (Madison, 2003). Skin is composed of three structural layers including: the epidermis, the dermis and the subcutaneous layer (Figure 1.1). Human skin also contains various appendages including sebaceous glands, which secrete an oily or waxy matter, called sebum, to lubricate and waterproof the skin and hair of mammals (Hunter, 2003). Sweat glands are found in almost every part of the skin and produce sweat that works to maintain the homeostasis of the body. Widespread vasculature in the dermis helps to regulate body temperature, to deliver oxygen and nutrients, and to remove toxins and waste products (Hunter, 2003).

![Figure 1.1. Skin Structure. Adapted from (www.MayoClinic.com), December, 2014](image_url)
1.1.1. Epidermis

The epidermis is the outermost skin layer contains epidermal cells such as, keratinocytes, Merkel cells, melanocytes and Langerhans cells (Figure 1.1). Keratinocytes are the main cells of the epidermis, these cells are connected to each other by proteins called desmosomes. The epidermis layer is formed during keratinocytes differentiation (Green et al., 1982, Eckert, 1989). These are four layers arranged as rows started from the stratum basale layer which lies above the basement membrane of the skin (Figure 1.2). Stratum spinosum is the second layer situated above the basale layer. Stratum granulosum is the third layer in the skin named based on the granules that form it. These granules supports the terminal stage in keratinocyte differentiation to the stratum corneum which is the outermost layer in the epidermis (Figure 1.2). Furthermore, the stratum lucidum is a thin layer found in the thick epidermis. This layer represents a conversion from the stratum granulosum and stratum corneum and is not usually seen in thin epidermis (Figure 1.1).

![Figure 1.2. Epidermal differentiation. Adapted from (www.jcs.biologists.org), December, 2014.](image-url)
1.1.1.1. Epidermal layers

1.1.1.1 Stratum basale

*Stratum basale* is the innermost layer of the epidermis (Figures 1.1 and 1.2), located next to the dermis. Keratinocytes are the main cells in this layer where they are attached to the basement membrane by hemidesmosomes (Heyden *et al.*, 1992). This layer also contains another cells such as Langerhans, Merkel cells and melanocytes. Melanocytes are responsible for melanin production. Melanin is a pigment that provides protection against ultraviolet radiation (UVR) (Prota, 1997, Kobayashi *et al.*, 1998a). It is well known that the black skin is less susceptible to UVR than the white skin because the distribution and rate of production of melanin is different in white and black skin (reviewed in (Brenner and Hearing, 2008)).

1.1.1.2. Stratum spinosum

The *stratum spinosum* is found on top of the basal layer (Figures 1.1 and 1.2) and consists of two to six rows of keratinocytes. Keratins produced by keratinocytes in this layer are serves to form cytoplasmic protein termed tonofilaments (Mottaz and Zelickson, 1975). This protein is required for the formation of the desmosomes that connect the cell membranes of adjacent keratinocytes together. The continuation of epidermal differentiation makes the cells in the upper layers of *stratum spinosum* to become flat and long as step toward the *stratum granulosum* formation.

1.1.1.3. Stratum granulosum

*Stratum granulosum* is formed after the *stratum spinosum* layer during keratinocytes differentiation (Figures 1.1 and 1.2.). *Stratum granulosum* is a thin layer in the epidermis contains on protein called keratohyalin assists to bind the keratin filaments together. As the keratinocytes continue in their differentiation, they lose the nuclei and organelles to form non-viable corneocytes in the *stratum corneum*. Also, during this time keratinocytes secrete lamellar granules that containing lipids and proteins into the
extracellular space to form hydrophobic lipid envelope works as the skin barrier (Freinkel, 2001). Finally, the cornified cells are formed as the keratinocytes lose their ability to differentiate in this layer (Ishida-Yamamoto and Iizuka, 1998).

### 1.1.1.1.4. Stratum corneum

Epidermal differentiation is end up with the formation of the *stratum corneum* layer (Figures 1.1 and 1.2). The *stratum corneum* functions as a barrier to protect the skin from environmental insults or any harmful substances or organisms. The *stratum corneum* in addition regulates water loss from the body and the water plays an important role in the integrity of *stratum corneum* barrier. The barrier nature of the *stratum corneum* depends on the proteins and lipids in the lamellar bodies secreted from the keratinocytes. According to Wilkes, (1973) the *stratum corneum* is composed of about 75-80% protein, 5-15% lipid (Wilkes *et al.*, 1973). The protein is found in the keratinocytes and it is about 70% alpha-keratin and 10% beta-keratin while, 5% is proteinaceous cells envelop and the 15 % remaining are formed of enzymes and other proteins. While, phospholipids do not exist in the *stratum corneum* layer (Lampe *et al.*, 1983), fatty acids, cholesterol, cholesterol sulfate and sterol/wax esters represents the main lipids in this layer.

### 1.1.1.2. Epidermal cells

#### 1.1.1.2.1. Keratinocytes

Keratinocytes are the major type of cell in the epidermis. They constitute approximately 95% of all cells found in the epidermis (Eckert, 1989). In the *stratum basale* keratinocytes exist as either stem cell-like cells i.e. they generate progeny by mitosis some of which retain stem cell characteristics, or transiently amplifying cells i.e. they replicate with a higher frequency than the stem cell-like keratinocytes but are only capable of limited population doublings (Eckert, 1989). Once a keratinocyte becomes fully committed to differentiation it will detach from the basement membrane,
differentiate and ultimately cease to proliferate as it migrates toward the skin surface, where it will be sloughed off as a fully differentiated dead cornified cell (Figure 1.1) (Eckert, 1989). As the keratinocyte migrates it expresses a progressive array of different keratins, which are a family of tough and insoluble structural proteins that help the skin protect from the external environment and help maintain its structure (Heyden et al., 1992). Keratinocytes in the basale layer of the epidermis produce keratin type 5 and type 14 (Heyden et al., 1992) and throughout the epidermis they are joined to one another by intercellular junctions known as desmosomes.

As the keratinocyte differentiate to the stratum spinosum layer its replicative potential is reduced as its ability to produce keratin increases and its ability to produce different types of keratin changes (Heyden et al., 1992). In addition to the production of keratin, the keratinocytes also produce keratinosomes. At this stage of terminal differentiation the keratinocytes mainly produce keratin types 1 and 10 (Heyden et al., 1992) (Figure 1.1.). Once the keratinocyte reaches the stratum granulosum it can no longer undergo cell division and instead undergoes cornification (Eckert, 1989). Cornification is due to the presence of keratohyalin granules and filaggrins (Candi et al., 2005). The epidermis is between 5mm and 150mm thick (depending on body site) and as mentioned before, it takes about 30 days for a keratinocyte to differentiate from the stratum basale to the stratum corneum (Eckert, 1989). Calcium is required for a keratinocyte to differentiate from the stratum spinosum and from the stratum granulosum to the stratum corneum (Bikle, 2004), while protein kinase-C (PKC) is needed for the keratinocytes to differentiate from the stratum spinosum to the stratum granulosum (Denning, 2004) (Figure 1.1.). The intracellular concentration of calcium increases as keratinocytes differentiate from the stratum basale to the stratum corneum, allowing the cells to increase the number of their intracellular connections (Denda et al., 2003). Keratinocytes have been reported to participate in immunological and inflammatory processes (Forsslind et al., 2004). It is well known that keratinocytes produce cytokines such as interleukins (IL) and tumour necrosis factor-alpha (TNF-α), which modulate the inflammatory response to UVR exposure (Tsatsanis et al., 2006). After UV radiation, TNF-α and IL-1α are up-regulated in keratinocytes, activates NF-κB transcription factor.
This transcription factor controls expression of around 90 genes involved in inflammation including cyclooxygenase-2 (COX-2) (Tsatsanis et al., 2006). In contrast, UVR increases expression of the anti-inflammatory cytokine; interleukin-10 (IL-10) which in turn decrease other pro-inflammatory cytokines such interleukin-1 (IL-1) and interleukin-6 (IL-6) (Rivas and Ullrich, 1992).

1.1.2.1. Immortalized HaCaT keratinocytes

Life of normal human somatic cells is limited and their growth is ceased irreversibly after certain number of divisions through a process called cellular senescence, or replicative senescence. However, cellular immortality or unlimited proliferation can be induced under culture conditions. Cellular immortality can be reproducibly induced by certain DNA viruses such as simian virus 40, adenovirus types 5 and 12, and human papilloma virus types 16, 18, 33 (Rhim et al., 1985, Rhim et al., 1986). The HaCaT line is a spontaneously immortalized human keratinocyte cell line which developed through long-term culture of normal human adult skin keratinocytes at reduced calcium concentration and elevated temperature (Boukamp et al., 1985). It was designated as HaCaT (Ha = human adult, Ca = calcium, T = temperature) to indicate its origin and the initial culture conditions. Although, this cell lines has genetic abnormalities, such as mutations in p53 (Lehman et al., 1993), it is still widely used and important for many studies of skin biology and the pathogenesis of skin-related diseases.

1.1.2.2. Melanocytes

In addition to keratinocytes, the stratum basale contains other specialised cell types such as the melanocytes (Figure 1.2.) (De Luca et al., 1988). Melanin pigment is synthesized within the melanocytes and then transferred to surrounding keratinocytes (Prota, 1997). Melanin absorbs ultraviolet light and protects skin from solar radiation (Prota, 1997). In addition to its photoprotective role, epidermal melanin also regulates cytokines activity where synthetic melanin was found to inhibit pro-inflammatory cytokines include TNF, IL-6 and IL-10 (Mohagheghpour et al., 2000). Moreover, human melanocytes serves as phagocytes against microorganisms (Le Poole et al., 1993).
Also, epidermal melanocytes were found to produce PGE$_2$ under basal condition (Nicolaou et al., 2004). Whereas, this eicosanoid was increased after UVB exposure (Gledhill et al., 2010).

Two forms of melanin are found: eumelanin is the more common brown/black form, whereas the less common phaeomelanin is red or yellow. Melanocytes make surface contact with adjacent keratinocytes through dendritic connections, and this allows the pigment granules to pass from the melanocytes to the keratinocytes (Kobayashi et al., 1998b). Melanocyte presence appears to be inducible with chronic exposure to light increasing the relative proportion of the pigment-forming cells within the basal layer.

There are equal numbers of melanocytes in a given body site in darker and lighter skin types, but darker-skinned people have more active and efficient melanocytes (De Luca et al., 1988).

1.1.1.2.3. Langerhans cells

Langerhans cells (LCs) are the main antigen presenting cells in the epidermis (Steinman et al., 1995), and it has been reported to be involved in the skin’s immune responses (Steinman et al., 1995). In addition, LCs reported to have a role in inflammatory conditions (Kripke et al., 1990, Hemmi et al., 2001). LCs are also found within the stratum basale (Figure 1.2) and in hair follicles (Steinman et al., 1995). They were described in 1868 by Paul Langerhans and they are derived from bone marrow. They are dendritic cells and through these processes connect to keratinocytes (Steinman et al., 1995). Although LCs are not efficiently phagocytic, they may present the antigens to lymphocytes in the lymph nodes (Steinman et al., 1995).

When compared to other membranes of the body, the skin comes into contact with many potential antigens and hence the LCs play an important role in conditions such as allergic contact dermatitis. As they are not linked to other cells by desmosomes, they can move and migrate from the skin to the immune system (lymph nodes) (Kripke et al., 1990). It is thought that they are able to internalise and process foreign antigens and transport them to the skin lymph nodes where cutaneous immune responses are
initiated (Cumberbatch et al., 2003). Upon exposure to UVR, LCs migrate to the lymph nodes where they are scavenge by cells of the immune system mainly Th cells for the presence of antigen (Allan et al., 2006, Dandie et al., 2001). LCs migration and maturation is mainly controlled by cytokines that are secreted by LCs and those secreted by surrounding keratinocytes (Kripke et al., 1990, Griffiths et al., 2005).

1.1.2.4. Merkel cells
They are large cells found in low number in the stratum basale layer of the epidermis (Sidhu et al., 2005) (Figure 1.2.). Merkel cells first described in 1875 by F. S. Merkel. They are found in greatest numbers around the touch-sensitive sites of the body, such as the lips and fingertips (Iggo and Muir, 1969). Merkel cells are associated with an afferent nerve terminal, forming a structure known as a Merkel cell-neuron complex (Saxod, 1996). Merkel cells also contain sensory receptors called merkel receptors found in touch-sensitive areas of the skin surfaces such as hands and feet (Halata et al., 2003).

1.1.2. Dermis
Dermis (Figure 1.2) is the second layer of the skin contains intensive connective tissues and the dermal fibroblasts formed the majority in this layer (Fleischmajer et al., 1995). It also contains lymphatic vessels, hair follicles, sebaceous, sweat glands sensory neurones, motor neurones and a tough, supportive cell matrix (Wilkes et al., 1973, Sorrell et al., 1996). Two layers comprise the dermis: thin papillary layer and thicker reticular layer. The papillary dermis lies below and connects with the epidermis (Sorrell et al., 1996). It contains thin loosely arranged collagen fibres, thick bundles of collagen run parallel to the skin surface in the deeper reticular layer, which extends from the base of the papillary layer to the subcutis tissue (Sorrell et al., 1996). Fibroblasts in this layer produce collagen, elastin and structural proteoglycans, together with immunocompetent mast cells and macrophages (Fleischmajer et al., 1993). Collagen fibres make up 70% of the dermis provide its strength and toughness whereas elastin maintains normal
elasticity and flexibility and proteoglycans provide viscosity and hydration (Sorrell et al., 1996).

1.1.2.1. Dermal cells

1.1.2.1.1. Fibroblasts

The presence of dermal fibroblasts within the dermal tissues is vital, as dermal fibroblasts are responsible for the production and structuring of extracellular matrix components, or ECM, within the skin (Ross et al., 1970). Further, dermal fibroblasts communicate among themselves and with different cells to regulate the physiology of the skin (Ross et al., 1970). Dermal fibroblasts constantly produce substances which are then used to make up ECM, including various types of collagen; fibronectin and types of elastin, integrin, proteoglycan and laminin, thus supporting the connective tissues in the skin (Ross et al., 1970, Fleischmajer et al., 1993, Sorrell et al., 1996). Due to this function, the dermal fibroblast is viewed as a central element in recovery from wounds (Ross et al., 1970). Dermal fibroblasts are found both in the reticular dermis and papillary dermis, and culturing these two subgroups has revealed variation in the features of each (Azzarone and Macieira-Coelho, 1982, Schafer et al., 1985, Sorrell et al., 1996, Sorrell et al., 2004).

The area available for molecules which display solubility to be transferred to the epidermal layer, as well as for the occurrence of epithelial–mesenchymal interaction is expanded by a large degree by the dermal papillae epidermis (Schafer et al., 1985, Sorrell et al., 1996). The dermal reticular layer is located between the superficial and the deep vascular plexi, and separates the hypodermis and dermis (Azzarone and Macieira-Coelho, 1982, Schafer et al., 1985). The reticular dermis is penetrated by hair follicles as well as the cells of the dermis with which they are linked, which frequently end within the adipocyte-rich hypodermal layer (Azzarone and Macieira-Coelho, 1982). Division takes place at greater pace in papillary fibroblasts compared with their reticular counterparts (Azzarone and Macieira-Coelho, 1982, Schafer et al., 1985, Sorrell et al., 1996, Sorrell et al., 2004). Biological study concerning the skin frequently utilises 46BR.IN fibroblasts, and are produced for this end by taking them from a subject who
has hypo-gammaglobulinemia before immortalizing them via plasmid pSV3neo, transfection to express the T-antigen SV40, as described by Arlett et al. (1988).

The skin’s epidermal layer is bound with the dermis beneath through means of a basement membrane composed of multiple molecules structured in a complex manner (Burgeson and Christiano, 1997, Aumailley and Rousselle, 1999). Fibroblasts and keratinocytes work together to create this membrane, which is distinguishable from other tissues of the skin (Fleischmajer et al., 1993, Marinkovich et al., 1993, Smola et al., 1998, Moulin et al., 2000). At the dermal-epidermal junction or DEJ, collagens IV and VII are excreted from keratinocytes and fibroblasts, while laminin-1 and laminin-5 are excreted from fibroblasts and keratinocytes respectively, to form a linear array (Marinkovich et al., 1993).

1.1.2.1.2. Mast cells

The mast cells of the skin lie deeper within the tissues, being found in the dermis, where they are grouped around blood vessels and nerves (Eady et al., 1979). Mast cells have an important role in inflammatory and allergic reactions such as allergic dermatitis, and they secrete bioactive mediators such as histamine, cytokines and eicosanoids such as prostaglandin D₂ (PGD₂) (Dawicki and Marshall, 2007, Wedemeyer et al., 2000). In addition they have been reported to control local blood flow and angiogenesis (Marks et al., 1986). These cells are affected by UVR which induces mast cells migration from skin to draining lymph nodes (Byrne et al., 2008).

1.1.2.1.3. Subcutaneous layer

The subcutaneous tissue (Figure 1.2) is the layer between the dermis and the fascia (Cross and Roberts, 1993). The fat tissue acts to preserve neutral fat, cushion against external physical pressure, retain moisture and generate heat (Cross and Roberts, 1993). The subcutaneous tissue is largely composed of fat cells, accumulated fat cells separated by the connective fibroid fat septum are called fat lobules (Wilkes et al.,
Fibre bundles produced in the dermis and firmly connected with the fascia and periostea through the subcutaneous tissue are found throughout this region (Cross and Roberts, 1993). These fibre bundles are called retinaculae cutis, and they strengthen the connection between the dermis and deeper tissues (Cross and Roberts, 1993).

1.2. Ultraviolet radiation and its effects on cells

The wavelength of the sunlight radiation is divided into three main regions of wavelengths (Figure 1.3). Ultraviolet (UV), visible, and infrared. UV radiation includes the wavelengths from 200 to 400 nm, these wavelengths are just shorter than those of visible light (400-700 nm). It is then characterized further UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm) reviewed in (Matsumura and Ananthaswamy, 2004). UVC from sunlight is virtually completely screened out by the earth’s atmosphere ozone layer. UV light is essential for normal skin health and a suitable dose of UVB radiation (280–320 nm) is necessary for processes including synthesis of vitamin D synthesis in the skin. However, UVR has also harmful effects on skin cells, these effects will be discussed in more details below.

*Figure 1.3: UV radiation spectrum* (Adapted from www.globalspec.com) December 2014.
1.2.1. Effects of ultraviolet radiation on human skin

Excessive UV radiation penetrates the epidermis and dermis and interferes with normal skin function, causing a number of health disorders. UVB is the most harmful type of UVR, it has been reported to be responsible for erythema (sunburn), which is the visual effect of UVR on the skin, usually characterised by dilatation of the dermal blood vessels and then increases the blood flow, consequently leading to skin redness (Diffey, 1991). This is apparent from about 4 hours after exposure and is maximal at about 24 hours (Young et al., 1996, Rhodes et al., 2009). An immediate tan is produced at between 5 and 10 minutes of the skin being exposed to ultraviolet radiation, and this type of tan fades after twelve hours. By contrast, a delayed tan appears as the result of melanin excreted by stimulated melanocytes and may last from several weeks to several months (Diffey, 1991). Erythemal reactions vary based on the amount of UV radiation exposure, the spectrum within which it is emitted and the individuals’ skin. Potential for burning from sun exposure is frequently measured through observing the subject’s minimal erythema dose or MED. In addition, multiple-occasion UVR exposure can lead to epidermal hyperplasia causing the cells of the epidermis to hyperproliferate and this layer to thicken. At between 1 and 3 weeks, the stratum corneum becomes three times thicker, and at 7 weeks, may be up to 7 times as thick as originally. However, between 4 and 8 weeks after sun exposure ends, thickening is completely reversed. The thicker skin produced with frequent ultraviolet exposure protects against damage from that further exposure (Diffey, 1991, Soter, 1990).

For certain instances, burning from sun exposure is linked to skin alterations, which may include for example the presence in the dermis of inflammatory cells (Gilchrest et al., 1983, Hawk et al., 1988) the occurrence of the protein p53 (Burren et al., 1998), and the presence of burned apoptotic cells in the epidermal layer (Sheehan and Young, 2002). Further, skin which was exposed to UVB radiation could display immunosuppression (Novakovic et al., 2001) as well as melanogenesis (Sheehan et al., 1998, Sheehan et al., 2002). In general, two pathways have been proposed for damage caused by cytotoxicity disrupting cells’ regular functioning and linking UVB exposure to carcinogenesis in the skin’s tissues. Exposure to UVB can produce damage directly
incurred by DNA through the forming of atypical covalent bonds joining pyrimidine bases next to each other within molecules of DNA in an excited state (Young et al., 1998). Where these anomalies are not destroyed via the pathway for nucleotide excision repair prior to the DNA being further replicated (Young et al., 1996, Bykov et al., 1999, Budden and Bowden, 2013), they disturb base pairs, and produce mutations which cause melanomagenesis (Budden and Bowden, 2013).

Secondly, UVB exposure produces oxidative stress for normal cells within the skin, through leading to production of reactive oxygen species (ROS) (Lee et al., 2013a), which cause harm to structural elements within cells, such as to membranes, lipids, DNA and proteins (Waster and Ollinger, 2009). The eventual result of this includes photoaging (Pillai et al., 2005) and photodamage (Marrot and Meunier, 2008), as well as photocarcinogenesis (Halliday, 2005).

Similar to UVB, UVA can also cause DNA damage primarily through the generation of ROS (Kvam and Tyrrell, 1997). However, UVA and UVB have been found to stimulate the production of melanin and induce apoptosis of melanocytes and keratinocytes an effect that has been recognised as a protective mechanism in the skin (Bowen et al., 2003). UVB radiation is mostly absorbed by the epidermis and upper dermis whereas, UVA, with its longer wavelength can penetrate deeper through the epidermis to reach the dermis (Costin and Hearing, 2007).

### 1.3. Omega-3 polyunsaturated fatty acids

Any fatty acid composed with over three or more double bonds is known as a polyunsaturated fatty acid, PUFA, or long-chain polyunsaturated fatty acid, and includes more than 17 atoms of carbon. For omega-3 polyunsaturated fatty acids, the omega (n) element of the name represents the methyl terminal, while 3 denotes the position of the final double-bond at the same end. There are 2 principal omega-3 polyunsaturated fatty acids, namely C22:6 n-3 docosahexaenoic acid or DHA, and C20:5 n-3 eicosapentaenoic acid or EPA. These are generally contained in the diet through oils from fish: for example from salmon, sardine or mackerel and sardines. Meanwhile, C18
n-3 PUFA α-linolenic acid or ALA, which is the parent to omega-3, can be obtained from oils of vegetal origin and humans can use this to synthesise both DHA and EPA. The advantages of these fatty acids for health are considered various (Wall et al., 2010). Research suggests that omega-3 PUFA enhance the health of the heart and circulatory system (De Caterina, 2011) as well as improving rheumatoid disorders (Bhangle and Kolasinski, 2011). Further, recent research suggests an effect of these fatty acids against cancer: specifically in combatting colorectal cancers or CRCs (CRC) (Cockbain et al., 2012).

Polyunsaturated fatty acids act in the regulation of a variety of processes, such as clotting of the blood and blood pressure, as well as nervous and brain development and function (Das, 2006). Further, these fatty acids are an element in inflammation response regulation via the creation of eicosanoids, which are powerful in terms of their mediating function (Das, 2006, Calder, 2006). Neither linoleic acid (LA, C18:2n-6), which is used in building n-6 UPFA, nor α-linolenic acid (ALA, C18:3n-3), which is necessary to create omega-3 polyunsaturated fatty acids, can be produced in humans, and thus must be provided by dietary means (Burr and Burr, 1929).

1.3.1. Metabolism of essential fatty acids in human skin

In 1929 Burr and Burr stated that there are two fatty acids that cannot be produced by mammalian cells. These are Linoleic acid (LA; 18; 2n-6) and α-linolenic acid (ALA; 18: 3n-3), thus, termed essential fatty acids (Burr and Burr, 1929). LA is found in plant oils such as sunflower, safflower, and corn oils, cereals, animal fat, and wholegrain bread, while ALA is abundant in green leafy vegetables, flaxseed, and rapeseed oils. Although mammalian cells cannot synthesize LA and ALA, they can metabolize them into more physiologically active compounds by the introduction of further double bonds (desaturation) via Δ5 and Δ6 desaturases, and by lengthening the acyl chain (elongation) via elongases (Figure 1.4). LA has been demonstrated to be the most abundant PUFA in human skin (Chapkin et al., 1986, Wertz, 1992). LA has reported to play a role in the epidermal water barrier function (Hansen and Jensen, 1985). LA also
undergoes metabolism to produce different metabolites, for example, incubation of 15-lipoxygenase (15-LOX) prepared from skin epidermis, metabolizes LA to 13-hydroxyoctadecadienoic acid (13-HODE) which is the major metabolite of LA in the epidermis (Ziboh et al., 2000). Elongation and desaturation of LA and ALA produces eicosapentaenoic acid (EPA; 20: 5n-3), arachidonic acid (AA; 20: 4n-6), and docosahexaenoic acid (DHA; 22: 6n-3). Elongation of gamma-linolenic acid (GLA; 18: 3n-6) forms dihomo-gamma-linolenic acid (DGLA; 20: 3n-6). At high concentrations DGLA is metabolized by epidermal cyclooxygenases (COX) into prostaglandins of 1-series such as PGE₁ and also by 15-LOX to 15-hydroxyeicosatrienoic acids such as 15-HETE (Ziboh et al., 2000). However, EPA and DHA have also been shown to be metabolized by epidermal 15-LOX to predominantly monohydroxylated metabolites such as 15-hydroxyeicosapentaenoic acid (15-HEPE) and 17-hydroxydocosahexaenoic acid (17-HoDHE) respectively (Miller et al., 1990, Miller et al., 1991). The n-6 PUFA AA represents about 9% of the total fatty acids in humans skin (Vroman et al., 1969). AA metabolised in the epidermis by the COX pathway to produce different types of prostaglandins (PGs) such as PGE₂, PGF₂α and PGD₂ (Vroman et al., 1969). Also, AA metabolised by 15-LOX to generate 15-hydroxyeicosatetraenoic acid (15-HETE) (Ziboh et al., 2000). In contrast, 5-LOX activity in the epidermis has reported to be insufficient to transform AA to leukotriene (LTB₄) (Iversen et al., 1993). Finally, it is well documented how the essential fatty acids are important in the epidermis, thus any deficiency of them will have an effect on skin health (Hansen and Jensen, 1985).
Figure 1.4: Metabolism of omega-9, 6 and 3 fatty acids. Adapted from (Wall et al., 2010). The essential fatty acids Linoleic and alpha-Linolenic acids are not synthesized by mammalian cells and need to be obtained through diet.
1.3.2. Polyunsaturated fatty acids and impact upon eicosanoids production

The eicosanoid group encompasses prostaglandins or PG, leukotrienes or LI, prostacyclin or PGI₂ and thromboxanes or TX. These find their precursor in 20-carbon PUFA; principally AA. They are significant in mediating and regulating functions for inflammatory processes (Tilley et al., 2001), and in modulation of inflammation as a response in terms of length of time for which inflammation persists, and how strong these processes are (Tilley et al., 2001, Kinsella et al., 1990). Despite this importance, generally, the impact physiologically which follows inflammation is based upon which cells are located in that area, how the response is caused, timing for production of eicosanoids and numbers of the various eicosanoids as related to the area involved, as well as how sensitive the region is where the eicosanoids are produced is at tissue and cellular level (Calder, 2008). Polyunsaturated fatty acids of the n-3 and n-6 varieties compete PUFA in generating eicosanoids via COX enzymes and LOX enzymes: however, as inflammatory cells generally have a significant percentage of AA, this generally forms the principal substrate in synthesising eicosanoids (Calder, 2001, Simopoulos, 2002). Within the membranes of cells, phospholipases release AA, and in particular phospholipase A₂ with free AA thereafter providing substrates for LOX and COX. As COX metabolises AA, 2-series PGs as well as 2-series thromboxanes or TXs are produced. Significant quantities of prostaglandin F₂α (PGF₂α) and prostaglandin E₂ (PGE₂) are generated by monocytes and macrophages, while some PGE₂ is created by neutrophils and prostaglandin D₂ or PGD₂ is generated by mast cells.

When AA is metabolised via the 5-LOX path, this leads to production of derivative of hydroxy hydroperoxy. These include 5-HPETE and 5-HETE, as well as 4-series LTs and, leukotrienes A₄ (LTA₄), B₄ (LTB₄), C₄ (LTC₄), D₄ (LTD₄) and E₄ (LTE₄). LTB₄ is generated by monocytes, neutrophils and macrophages. Meanwhile, mast cells, eosinophils and basophils generate LTC₄, LTE₄ and LTD₄. The Arachidonic acid gives the basis for so-called proinflammatory eicosanoids (Bagga et al., 2003), which include interleukin-6 (IL-6) as generated by macrophage cells in response to PGE₂, and the functions of which include dilation of vascular channels and generation of pain (Tilley et al., 2001, Bagga et al., 2003). Neutrophils meanwhile can be activated by LTB₄, which
also impacts leukocytes by acting strongly as a chemotactic agent, as well as stimulating macrophage generation of cytokines involved in inflammation including tumour necrosis factor alpha, or TNFα, IL-1 beta, or IL-1β and IL-6 (Tilley et al., 2001). While the status of proinflammatory mediator is frequently applied to eicosanoids derived from AA however, these substances are also significant in modulation of immune responses because of their ability to interact with leukocytes in a manner which displays considerable complexity the initial inflammatory processes (Tilley et al., 2001, Simopoulos, 2002). Despite these benefits, where such substances are present in excess, the tissue local to their production may be harmed and conditions involving inappropriate inflammatory response, as well as thrombosis, are partially caused by this mechanism (Simopoulos, 2003).

LOX metabolises DHA to produce docosanoids, as well as resolvins (Hong et al., 2003). Resolvins have a mediatory function, act locally and are endogenous: they act against inflammation and help to regulate immune response (Serhan et al., 2002, Serhan et al., 2007, Schwab et al., 2007). As concerns the cell, functions encompass reduction in neutrophils infiltrating as well as regulating reactive oxygen species and the cytokine-chemokine axis, and effecting reduction in inflammation-response (Serhan et al., 2000, Serhan et al., 2002).

Both LOX and COX metabolise EPA, creating disparate eicosanoid groups, namely HEPE, 3-series TXs and TXs, and 5-series LTs. As contrasted with AA-derived eicosanoids, those of EPA may not be so, or may act against inflammation (Bagga et al., 2003, Robinson and Stone, 2006). Variation in inflammatory effect between AA- and EPA-derived eicosanoids is exemplified by LTB4 in comparison with LTB5. LTB4 is stronger in producing inflammatory response than LTB5, due to its action as a chemotactic agent for neutrophils, which is greater by a factor of between 10 and 100 (Goldman et al., 1983, Lee et al., 1984). In addition, resolvin E1, which derives from EPA, is protective against colitis, as evidenced by studies of animals (Weylandt et al., 2007). Further, PGE2 is described in the literature as more powerful in inducing macrophage IL-6 generation in comparison with PGE3 (Bagga et al., 2003). Greater amounts of n-3 PUFA, including DHA and EPA ingested leads to increased
concentration of these substances within inflammatory cell membranes, as reported by Endres et al. (1989) and Yaqoob et al. (2000). Both DHA and EPA make up inflammatory cells in humans in dose-response relation to their intake (Healy et al., 2000, Rees et al., 2006) with this make-up able to reduce AA to some extent.

1.3.3. Omega-3 PUFA and inflammatory gene expression

In addition to their effects on the eicosanoids, n-3 PUFA exert their anti-inflammatory effect by changing the inflammatory gene expression through their effects on transcription factors such as NFκB and peroxisome proliferator-activated receptors (PPARs) (Calder, 2006, Calder, 2002). NFκB is a transcription factor that plays an important role in various inflammatory signalling pathways. It controls several cytokines such as IL-1, IL-2, IL-6, IL-12, TNF-α, chemokines like IL-8, monocyte chemoattractant protein-1, adhesion molecules and inducible enzymes such as the inducible nitric oxide synthase (iNOS) and COX-2 (Ghosh and Karin, 2002). EPA has been shown to block the activity of NFκB through decreased degradation of the inhibitory subunit of NFκB, IκB, in cultured pancreatic cells and human monocytes (Lo et al., 1999, Novak et al., 2003, Zhao et al., 2004). This has also been supported by the finding that transgenic mice that endogenously biosynthesize n-3 PUFAs from n-6 PUFAs are protected from colitis through a decrease in NFκB activity (Hudert et al., 2006).

PPARs are ligand-activated nuclear transcription factors that play important roles in cellular differentiation, cancer, inflammation, insulin sensitization, atherosclerosis and several metabolic diseases (Chawla et al., 2001). Ligands for PPARs are PUFAs, especially those of the n-3 family and their eicosanoid derivatives (Kota et al., 2005). When activated, PPARs bind to the PPAR-response element and repress or induce the transcription of target genes. PPARs have been shown to inhibit NFκB and therefore play an important role in several inflammatory processes (Poynter and Daynes, 1998, Ricote et al., 1999). EPA and DHA for instance inhibit lipopolysaccharide-induced activation of NFκB via a PPAR-γ-dependent pathway in human kidney-2 cells (HK-2) (Li et al., 2005).
1.3.4. The protective effect of n-3 PUFA in UVR induced skin inflammation

The harmful effects of UVR in the skin include acute effects such as erythema, inflammatory and immune modulatory changes (Young, 2006). Chronic effects eventually lead to photoaging and photocarcinogenesis (Taylor et al., 1990). Although, there are some strategies to protect against UVR-induced skin damage such as topical sunscreens (Rhodes, 1998), these strategies have been found to be inadequate. The n-3 PUFA, EPA and DHA are considered as a dietary component with a promising photoprotective effect against the deleterious effects of UVR. They are mainly present in fish oil, and have been shown to have a protective effect against photocarcinogenesis in mice (Black et al., 1992). Supplementation with fish oil also reduces UVB-erythemal sensitivity in humans (Rhodes et al., 1994), by inhibition of UVB-induced PGE2 levels in the skin (Rhodes et al., 1995). Additionally, n-3 PUFA have been reported to improve some inflammatory diseases, including psoriasis (Mayser et al., 1998) and rheumatoid arthritis (Kremer, 2000), and can inhibit tumor formation (De Vries and van Noorden, 1992).

1.4. The endocannabinoid system

The major psychotropic component of the cannabis sativa plant, Δ9-tetrahydrocannabinol (THC) was discovered in 1964 (Gaoni and Mechoulam, 1964). After more than 20 years the first THC receptor was identified in the mammalian brain and named cannabinoid receptor type-1 (CB1) (Devane et al., 1988). Two years later and then the second G-protein-coupled receptor (CB2) was cloned (Matsuda et al., 1990). Whilst CB1 was shown to be extremely abundant in the brain, and hence suggested to be responsible for THC psychoactivity, CB2 was expressed in its highest levels in immune cells. The cloning of the cannabinoid receptors opened the way for more research to identify endogenous lipid ligands termed endocannabinoids. The first endocannabinoid to be discovered was N-arachidonoyl ethanolamide (AEA, anandamide) (Devane et al., 1992), soon after that followed by the detection of the 2-arachidonoyl-glycerol (2-AG) which shows high affinity for CB1 and CB2 receptors (Mechoulam et al., 1995, Sugiura et al., 1995). Furthermore, more endocannabinoids
have been found in the last decade, including 2-arachidonyl-glycerol ether (noladin ether) (Hanus et al., 2001), N-arachidonoyl-dopamine (NADA) (Bisogno et al., 2000) and virodhamine (Porter et al., 2002). However, their pharmacological activity and metabolism has not yet been thoroughly investigated. Therefore, the N-acylethanolamine anandamide and the monoacylglycerols 2-AG are still the most studied endocannabinoids (Figure 1.5).

The endocannabinoids exert their biological functions by binding to or activating CB1 and CB2 receptors (Howlett et al., 2002). However, they may have different responses for example, AEA behaves as a partial agonist at both CB1 and CB2 receptors, but has higher affinity for the CB1 receptor (Howlett et al., 2002). Thus, AEA has been reported to have the same pharmacological functions to cannabinoids such as Δ9-THC, by activating the CB1 receptors in the brain (Smith et al., 1994). In addition, AEA shows potent immunomodulatory and anti-inflammatory activities by interacting peripherally with CB receptors (Stefano et al., 1996).

AEA is transported into cells by the AEA membrane transporter (AMT) and its action is terminated by the enzyme fatty acid amide hydrolase (FAAH). Whereas, 2-AG uptake occurs through the 2-AG membrane transporter (2-AGMT) (Hermann et al., 2006), 2-AG can be hydrolysed by FAAH but the main enzyme responsible for 2-AG degradation is monoacylglycerol lipase (MAGL) (Cravatt et al., 1996, Dinh et al., 2002).
Figure 1.5: Chemical structures of the Δ⁹-tetrahydrocannabinol (THC), N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). Adapted from (Bari et al., 2011).

1.4.1. Biosynthesis of N-arachidonoylethanolamide (AEA, anandamide)

Anandamide or AEA is produced from membrane phospholipids, having the precursor N-arachidonoyl phosphatidylethanolamine or NAPE. These phospholipid undergo hydrolysis and form AEA via an enzyme: N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) as shown in Figure 1.6 (Di Marzo et al., 1994, Guo et al., 2005). This may be simplistic however, since synthesis of AEA appears to encompass a variety of pathways, including AEA produced independently from NAPE-PLD, as concentrations of the end substance were unaffected by knockout of NAPE-PLD in a study involving mice (Liu et al., 2006). Studies in mice also revealed NAPE-AEA conversion from NAPE-PLD (Leung et al., 2006).
1.4.1.1. N-acyltransferases

AA gives the derivative AEA as ethanolamine, derivatives of N-acylethanolamine (NAE) produced from metabolising different fatty acids and include a large number with endocannabinoid functions, this includes DHEA. In biosynthesising NAE, initially phosphatidylethanolamines (PE) are N-acylated to created ethanolamine phospholipids which are N-acylated, including NAPE regulated via the Ca2+ dependent N-acyltransferase or NAT (Hansen et al., 2000, Cadas et al., 1997, Jin et al., 2007). Phosphatidylcholine (PC), 1-acyl-lyso-PtdCho and PE are utilised by NAT to form donors in terms of substrate, while the enzyme selects acyl groups from these located at sn-1 for extraction.

1.4.1.2. N-acyl phosphatidylethanolamine phospholipase D

N-acyl phosphatidylethanolamine-phospholipase D, or NAPE-PLD is a member of the zinc metallohydrolase group from the β-lactamase, being separate in functional and structural terms from PLD enzymes generating phosphatidic acid or PA by hydrolysing glycerophospholipids, including PC, which acts between cells as a signalling molecule (Liscovitch et al., 2000, Okamoto et al., 2004). The principle pathway for synthesising AEA as well as various NAEs is NAPE-PLD (Schmid, 2000, Hansen et al., 2000, Schmid and Berdyshev, 2002, Nakane et al., 2002). NAE being released from NAPE via NAPE-PLD forms stage two in the process of synthesising NAE (Okamoto et al., 2007). It is suggested that zinc is a component of NAPE-PLD which is vital to catalysis, while purified recombinant enzyme correlates specifically to NAPE, is virtually non-reactive when considering significant glycerophospholipids including PE and PC (Wang et al., 2006). From animal tissue, there is only one enzyme which controls direct generation of NAE as released by NAPE: NAPE-PLD. In terms of regulating transcripts, the Sp1 transcription factor is identified as contributing to baseline expression of NAPE-PLD in a regulatory capacity (Zhu et al., 2011).
1.4.1.3. Alternate pathways for anandamide biosynthesis

A range of enzymes are identified as having roles in biosynthesising AEA, such as secretory phospholipase A2 or sPLA2. This functions to hydrolyse NAPE, forming N-arachidonoyl lysophosphatidylethanolamine or lyso-NAPE, and this is capable of hydrolysation via lysophospholipase D (lyso-PLD), giving AEA independently of Ca2+ (see Figure 1.6) (Sun et al., 2004). Furthermore, αβ-hydrolase 4 or Abhd 4 may be capable of affecting lyso-NAPE or NAPE and thus creating glycerophospho-arachidonoyl ethanolamide or GP-AEA. This substance then acts on metal-reliant phosphodiesterases, resulting in the formation of AEA (see Figure 1.6) (Leung et al., 2006). Further, phospholipase C PLC can act in hydrolysation of NAPE, forming phosphoanandamide, and this in turn undergoes dephosphorylation via phosphatases including tyrosine phosphatase PTPN22, and AEA is produced in this manner, as show in Figure 1.6 (Liu et al., 2006). Further, in macrophages treated with LPS, there was no alteration in the level of AEA found for siRNA knockdown NAPE-PLD, PTPN22 and Abhd 4 (Liu et al., 2008). The formation of AEA via avenues other than those discussed above is considered to occur through free AA condensing alongside ethanolamine within a FAAH reversed reaction (Arreaza et al., 1997, Kurahashi et al., 1997), which may take place given adequate quantities of ethanolamine and AA (Katayama et al., 1999). Generation of AEA in this manner was achieved on a study on live mice in which part of their livers was removed (Mukhopadhyay et al., 2011). Furthermore, McCue et al. (2009) state that spontaneous generation of AEA is possible based on ethanolamine and arachidonoyl-CoA.
Figure 1.6. Schematic showing the various pathways of anandamide biosynthesis. Adapted from (Bisogno and Maccarrone, 2014).
1.4.2. Biosynthesis of 2-arachidonoyl glycerol

In using phospholipids to produce AA, a principle avenue was viewed as producing diacylglycerol lipase (DAGL) via the action of phospholipase C or PLC upon phosphatidylinositol (Prescott and Majerus, 1983). The same avenue is also recognised as central to 2-AG synthesis (Sugiura et al., 2006, Ueda et al., 2011), which takes place within postsynaptic neurons as a reaction to Gq/11-coupled receptors being stimulated and depolarised. Kano et al. (2009) report that the release of the resultant 2-AG allows mediation of retrograde synaptic suppression via CB1 receptors located in presynaptic terminals. The path described includes use of PLC in hydrolysing 2-arachidonoylphosphatidylinositol or PtdIns, forming diacylglycerol (DAG), which contains arachidonic acid, and subsequently undergoes hydrolysation via DAGL, forming 2-AG, as shown in Figure 1.7. The most abundant PtdIns molecule in mammalian tissues is 1-stearoyl-2-arachidonoyl-PtdIns is the most frequently encountered PtdIns within the tissue of mammals and allows 2-AG to be created in preference to different MAGs. The β subtype or PLC may be stimulated via Gq/11-coupled receptors (Fukami et al., 2010), with the implication that PLC is important for generating neurotransmitter-reliant 2-AG with mediation through Gq/11-coupled receptors within postsynaptic neurons. However, it is also possible to convert PtdCho to 2-arachidonoyl-DAG (Oka et al., 2005) and the same is demonstrated for PA by Bisogno et al. (1999b) with this forming 2-AG precursor. PtdIns also sets in motion a path in which lyso-PtdIns production via PLA1 leads to lyso-PtdIns-specific PLC releasing 2-AG (Ueda et al., 1993, Tsutsumi et al., 1994) (Figure 1.6), as shown in Figure 1.6. Moreover, arachidonoyl-lysophosphatidic acid or Lyso-PA can also generate 2-AG (Nakane et al., 2002).

DAGL is an enzyme linked with membranes and which hydrolyses DAG in a preferential manner at locus sn-1 (Okazaki et al., 1981, Chau and Tai, 1981). Cloning via cDNA demonstrates two genes, α and β, which have a high degree of relation to each other, as involved in DAGL in humans (Bisogno et al., 2003). A broad distribution pattern has been found for DAGLα within the tissues of both mice and humans: it is found in particularly strong concentrations within the nervous system of both animals, and in human pancreatic tissue. Further localisation to PLCβ, Gq/11-coupled receptors and
Gq/11α within certain surfaces of synapses and neurons (Kano et al., 2009). Mice in which DAGLα was present in insufficient quantities display significantly lowered 2-AG within the brain, which suggests a high degree of involvement for DAGLα as opposed to DAGLβ with the production of 2-AG (Gao et al., 2010, Tanimura et al., 2010).

Figure 1.7. Schematic showing the main steps in the biosynthesis of the 2-Arachidonoylglycerol. Adapted from (Bisogno and Maccarrone, 2014)
1.4.3. Catabolism of AEA and 2-AG

Subsequent to their generation endocannabinoids rapidly become inactive, as shown in Figure 1.8. AEA within the brain returns to neurons before undergoing hydrolysation via FAAH to form ethanolamine and AA (Glaser et al., 2005, Fowler, 2007). As well as this process, LOX, COX-2 and cytochrome P450 may also generate prostaglandin ethanolamines (PG-EA), or prostamides, by metabolising AEA (Karsak et al., 2007). Further, COX-2 is capable of metabolising 2-AG through PGH2-GE as an intermediate in the production of prostaglandin glycerol esters including glyceryl prostaglandins and PG-GE (Kozak et al., 2000). Transportation of 2-AG to cells is carried out either through diffusion or via 2-AG transporters (Hermann et al., 2006). The majority of 2-AG hydrolysation taking place in brain tissues is conducted by monoacylglycerol lipase (MAGL). This substance allows 2-monoglycerides and 1-monoglycerides to be converted to glycerol and fatty acid (Dinh et al., 2002, Blankman et al., 2007). Further, it is possible that 2-AG is inactivated in a process which involves phosphorylating it with monoacylglycerol kinase (Nakane et al., 2002). As transportation, metabolic pathways and biosynthesis differ for 2-AG and AEA, it is suggested that each has its own mechanisms for regulation. Further, direct inhibition of 2-AG by AEA is described by De Petrocellis and Di Marzo (2009). Additionally, Fezza et al. (2008) report higher concentration of AEA within the striatum as effecting reduction in 2-AG’s impact upon GABAergic transmission.
1.4.3.1. **Fatty acid amide hydrolase**

FAAH is an amidohydrolase categorised within the amidase group of serine hydrolases, and has an association with membranes. FAAH is responsible for hydrolysing NAEs, including AEA, creating ethanolamine and specific fatty acids (Deutsch et al., 2002, Ueda et al., 2013).
McKinney and Cravatt, 2005). Research involving FAAH−/− mice showed that FAAH was important in the degrading process of several NAEs, including AEA (Cravatt et al., 2001, Clement et al., 2003). It is also involved in hydrolysation of oleamide and various fatty acid amides (Cravatt et al., 1996), as well as N-acyltaurine, as reported by Saghatelian et al. (2004). Hydrolysation of 2-AG occurs rapidly via FAAH (Goparaju et al., 1998), although studies have not considered FAAH to have a central importance in degradation of 2-AG within brain tissue (Goparaju et al., 1999, Blankman et al., 2007). Unlike rodent species studied, human tissues show expression of a FAAH isozyme which has approximately 20% sequence identity when viewed at the level of amino acids (Wei et al., 2006). The isozyme is categorised as FAAH-2, as distinct from FAAH-1, or the first FAAH to be identified. FAAH-2 shows a different localisation to FAAH-1, being found on droplets of lipids as opposed to the endoplasmic reticulum. Further, Kaczocha et al. (2010) report that for FAAH-2, the N-terminal hydrophobic area of FAAH-2 is shown to be a sequence for localising the isozyme on lipid droplets, and this implies a distinct function of FAAH-2 in comparison with FAAH-1. Moreover a range of symptoms are suspected to include FAAH involvement, among which are neuropathic pain as well as depressed and anxious states. This has led to the expectation that substances which inhibit FAAH may present drugs which have an effect in these scenarios (Petrosino and Di Marzo, 2010). Thus, development of a number of specifically targeted FAAH inhibitors has occurred, such as for OL-135 (Lichtman et al., 2004), URB597(Kathuria et al., 2003), PF-3845 (Ahn et al., 2009) and PF-04457845 (Ahn et al., 2011).

1.4.3.2. N-acylethanolamine hydrolysing acid amidase

The initial identification of the lysosomal enzyme N-acylethanolamine hydrolysing acid amidase or NAAA took place in humans in CMK megakaryoblastic cells, as reported by Ueda et al. (1999). This was followed by its identification in rat subjects within various tissues including those of the lung (Ueda et al., 2001). When the enzyme was cloned via cDNA, it was identified as cysteine hydrolase from the N-terminal nucleophile hydrolase supergroup (Tsuboi et al., 2005, Tsuboi et al., 2007a, Ueda et al., 2010). The
enzyme was considered a protein similar to the lysosomal enzyme acid ceramidase in sequence homology (Hong et al., 1999). Acid ceramidase acts in hydrolysing ceramide, forming fatty acids and sphingosine. Despite this, sequence homology has not been observed for FAAH and NAAA. Based on the fact that NAAA is localised within lysosomes, it shows activity solely when pH is in the acid area of the spectrum, hydrolysing a range of NAEs but favouring PEA as shown in vitro. The NAAA glycoprotein in humans has 4 loci of N-glycosylation (Zhao et al., 2007, West et al., 2011). West et al. (2011) report that a recombinant form of NAAA is generated to form a proenzyme which is not active, undergoing conversion through autocatalytic cleavage of Phe125 and Cys126, forming a heterodimer based upon α and β elements and which displays catalytic activity. Expression of NAAA can be found within a variety of tissues in both human and rodent subjects, and is principally expressed within macrophage cells (Sun et al., 2005, Tsuboi et al., 2007b). NAAA is expressed to the greatest degree in humans within prostate tissue (Wang et al., 2008). Stimulation of NAAA has been achieved in vitro via non-ionic detergent, including Nonidet P-40 Triton X-100, as well as through dithiothreitol, which is an agent in reducing thiol. Nonidet P-40 may be replaced by phospholipids which contain choline or ethanolamine, such as sphingomyelin, PtdCho and PtdEtn, in endogenous stimulation, whereas dithiothreitol can be replaced with dihydrolipoic acid, which is α-lipoic acid in reduction (Tai et al., 2012).

1.4.4. N-acylethanolamines

N-acylethanolamines, or NAEs, are substances derived from fatty acids, and these present themselves within the body tissues of mammals (Schmid, 2000, Hansen et al., 2000, Schmid and Berdyshev, 2002, Nakane et al., 2002). Certain NAEs are shown to fulfil particular roles, including for example N-stearoylthanolamide (STEA), which is pro-apoptotic (van der Stelt et al., 2002). while N-palmitoylthanolamide (PEA) acts against inflammation, as well as having neuroprotective and analgesic properties, and being an agonist for peroxisome proliferator-activated receptor-α as opposed to CB1 and CB2 (Calignano et al., 1998, Smart et al., 2002, LoVerme et al., 2005). Further, N-
oleoylethanolamide or OEA is suggested to be anorexic by Rodriguez de Fonseca et al. (2001). Further, OEA is an agonist for a range of receptors, such as; G protein-coupled receptor GPR119, peroxisome proliferator-activated receptor-α and transient receptor potential vanilloid 1 (Pavón et al., 2010). Each of these receptors derive from membrane phospholipids. Phosphatidylethanolamine (PE) is converted to N-acyl phosphatidylethanolamine (NAPE) by means of Acyltransferases, and NAPE undergoes hydrolysis via NAPE-PLD, producing N-acyl ethanolamine (Hansen et al., 2000), which is further elaborated in Section 1.4.1., and as an instance of this, hydrolysing N-pamitoylphosphatidylethanolamide, a phospholipid precursor, is considered via NAPE-PLD is considered to be a principle avenue in biosynthesising N-palmitoylethanolamide, as proposed by Schmid et al. (1983). Various N-acyl chains for NAE are considered to be biosynthesised via transacylation–phosphodiesterase. Further, Sugiura et al. (1996) did not identify N-acyl species when catalysing reactions via N-acyltransferase, and nor did Wang et al. (2006) when using NAPE-PLD.

1.4.5. Endocannabinoids and their impact on the skin

The endocannabinoid receptors CB1 and CB2 have been found in human and murine skin cells such as cutaneous nerve fibres, mast cells, epidermal keratinocytes and cells of the adnexal tissues (Stander et al., 2005, Ibrahim et al., 2005, Blazquez et al., 2006, Telek et al., 2007, Casanova et al., 2003). In particular, CB1 is expressed on different types of the cutaneous nerves such as the large myelinated nerve fibres in the papillary dermis, also on the small nerve fibres that associate with hair follicles and on the nerve fibres in the epidermis (Stander et al., 2005). In addition, the presence of CB1 receptor has been reported in all dermal mast cells (Stander et al., 2005). CB2 has been found in the skin on large myelinated nerve fibre bundles of the superficial and deep reticular dermis, small unmyelinated nerves of the papillary dermis and occasionally on nerves of the epidermis (Stander et al., 2005), as well as in human SZ95 sebocytes (Dobrosi et al., 2008).
The CB receptors have been suggested to be involved in the differentiation of keratinocytes (Casanova et al., 2003). For instance, endocannabinoids were found to regulate human epidermal differentiation, probably via CB1-dependent mechanisms. Therefore, local administration of AEA inhibited the differentiation of cultured NHEK and HaCaT keratinocytes, as evidenced by the transcriptional downregulation of keratin 1, keratin 5, involucrin and transglutaminase-5 and suppression of the formation of cornified envelopes (Maccarrone et al., 2003b). In addition, activation of the CB receptors either by phytocannabinoids or these from synthetic sources has been demonstrated to inhibit the proliferation process in cultured transformed human epidermal keratinocytes (HPV-16 E6/E7) (Casanova et al., 2003). However, cellular growth of cultured NHEKs and non-tumorigenic human HaCaT and murine MCA3D keratinocytes was not altered by synthetic CB1 and CB2 agonists (Casanova et al., 2003).

AEA and 2-AG have been found in murine epidermal cells (Berdyshew et al., 2000) and in rodent skin (Calignano et al., 1998). In human skin, AEA and 2-AG were detected in organ-cultured hair follicles (Telek et al., 2007) and SZ95 sebocytes (Dobrosi et al., 2008). Endocannabinoids also have been suggested to play a regulatory role in the human pilosebaceous unit; which control a wide range of the biological functions of the skin from stem-cell supply through immunomodulation to cytokine production (Roosterman et al., 2006, Paus et al., 2006a, Paus et al., 2006b). For example; endocannabinoids might act as a regulators of human hair growth. Consistently with this idea, CB1 receptor antagonists have been shown to induce hair growth in mice (Srivastava et al., 2009).

As it was mentioned above endocannabinoids are involved in the regulation of the skin cells growth in vivo. Local administration of synthetic CB1 and CB2 agonists induce growth inhibition of malignant skin tumors generated by intradermal inoculation of tumorigenic PDV.C57 mouse keratinocytes into nude mice (Casanova et al., 2003). This growth inhibition was accompanied by enhanced intra-tumor apoptosis and impaired tumor vascularization. Conversely, CB receptors and the related signalling pathways have been suggested to be involved in the promotion of in vivo skin carcinogenesis.
(Zheng et al., 2008). By using CB1/CB2 double gene-deficient mice, the study demonstrated that absence of CB1 and CB2 receptors results in a marked decrease in UVB-induced skin carcinogenesis. They also found marked attenuation of UVB-induced activation of MAPKs and nuclear factor-κB was associated with CB1 and CB2 receptors deficiency (Zheng et al., 2008).

Endocannabinoids exert a protective role in allergic inflammation of the skin (Karsak et al., 2007). Also the same study found that mice lacking CB1 and CB2 or treated with antagonists of these receptors exhibited exacerbated allergic inflammatory response (Karsak et al., 2007). The existence of the endocannabinoid-mediated protection was also supported by a reduced allergic response in the skin of FAAH-deficient mice that has increased levels of AEA (Karsak et al., 2007). However, 2-AG levels were increased in skin with acute and chronic contact dermatitis (Oka et al., 2006) and the symptoms of skin inflammation were significantly reduced by CB2 antagonists (Oka et al., 2006). Moreover, the cutaneous inflammation of contact dermatitis was found to be decreased in CB2-deficient mice (Ueda et al., 2007), and similar suppression of the inflammatory response by orally administered CB2 antagonists was also observed (Ueda et al., 2007, Ueda et al., 2005).

Most of these studies focused on the existence of endocannabinoids and their CB1 and CB2 receptors in human skin cells and the role of these receptors on skin health and skin disorders. However, the role of these endocannabinoids in skin disorders, in particular these related to UVR is still waiting to be investigated. Specifically, it would be interesting to investigate the effects of UVR on endocannabinoid production by human skin cells. As the n-3 PUFA, EPA and DHA are considered as a dietary component with a promising photoprotective effect against the deleterious effects of UVR, the formation of n-3 PUFA, NEA is also interest.
1.5. Aims and objectives of the study

Exposure to UV radiation has a range of effects and can cause skin disorders such as erythema, sunburn, photoaging and photocarcinogenesis (Taylor et al., 1990, Young, 2006). It is clear that the predominant exposure to UVR may be occurs inadvertently under everyday circumstances (Godar et al., 2003). There are some protection strategies to save the skin from the harmful effects that may be initiated by UVR during times of intense exposure (Wingerath et al., 1998). For example, avoidance of sun exposure, wearing protective clothing or using the topical application of sunscreens. Although the use of topical protection sunscreens is considered as a part of skin cancer prevention plans (Moloney et al., 2002), this method of protection may affect the synthesis of vitamin D in the skin leading to disorders related to vitamin D deficiency such as reduced bone strength (Moloney et al., 2002). In addition to its action on vitamin D synthesis, sunscreens should follow standardized methods to apply its protection effect against UVB. For example, each cm² of skin should be covered by 2 mg of sunscreen (Ferguson, 1997). In reality, sunscreens are employed under nonstandardized conditions, and often topical application may be inadequate to confer optimal protection against UV radiation (Bech-Thomsen and Wulf, 1992, Krutmann, 2001, Diffey, 1996). Therefore, finding more effective protection approaches is still required.

Photoprotection by endogenous compounds provided from the diet is became one of the most promising approach in this field. In general, n-3 PUFA EPA and DHA have been considered as a dietary component with a promising photoprotective effect against the deleterious effects of UVR (Black et al., 1992, Rhodes et al., 1994, Rhodes et al., 1995). They exert their anti-inflammatory effects through competition with AA as substrates for COXs and LOXs enzymes, resulting in the formation of less active prostaglandins and leukotrienes (Lands et al., 1992). n-3 PUFA also reduces UVB-erythemal sensitivity in humans (Rhodes et al., 1994), by inhibition of UVB-induced PGE₂ levels in the skin (Rhodes et al., 1995). N-3 PUFA can also alter the levels of other PUFA acting as substrates for endocannabinoids and other NAEs (Kozak et al., 2002a, Kozak et al., 2002b, Kozak et al., 2004). n-3 PUFA therefore, could have an
effect on the endocannabinoid pathways in the skin cells and may lead to production of NAE such as N-stea-roylethanolamine (STEA) which has been reported to have pro-apoptotic activity (van der Stelt et al., 2002) and/or N-palmitoylethanolamine (PEA) that showed anti-inflammatory activity, analgesic and neuroprotective effects (Calignano et al., 1998, Smart et al., 2002, LoVerme et al., 2005). Manipulation of the endocannabinoid pathways through n-3 PUFA supplementation could be the major defense mechanism behind the protective effect of n-3 PUFA against UVR in the skin cells. Therefore, we will examine the hypothesis that n-3 PUFA particularly EPA and DHA may have a biological role in protecting human skin cells against the harmful effects of UVR through their effect on the formation of endocannabinoid and NAE or through their effect on the endocannabinoid metabolizing enzymes such as FAAH, NAPE-PLD, COX-2 and LOX. To address these questions we used two cell lines: HaCaT keratinocytes and 46BR.1N fibroblasts. Cells will be treated DHA and EPA in the presence and absence of UVR. AEA, 2-AG, NAE, NAPE-PLD, FAAH, COX-2 and 12-LOX and 15 LOX were investigated in nonirradiated and irradiated cells using Western blot for protein experiment and LC-MS/MS for lipid analysis. Moreover, through our collaboration in a clinical study, we will examine the effect of UVR on endocannabinoids and NAE found in circulation. Therefore, the specific objectives of this project were:

1. To study the effect of UVR and n-3PUFA on the formation of endocannabinoids and their congeners in HaCaT keratinocytes and 46BR.1N fibroblasts.
2. To explore the effect of UVR and n-3PUFA on the endocannabinoid metabolizing enzymes.
3. To assess the effect of UVR on the circulating levels of endocannabinoids and their congeners through analysis of serum samples.
CHAPTER 2: Materials and Methods

2.1. Cell culture and maintenance

2.1.1. Cell lines

The HaCaT human keratinocyte cell line was provided by Dr S. Britland, University of Bradford and the 46BR.1N human fibroblast cell line was purchased from The European Collection of Cell Culture (ECACC).

2.2. Cell culture

2.2.1. Materials

Hank’s balanced salt solution (HBSS, Cat No. C-40390) was purchased from Promo Cell, (Heidelberg, Germany). Dulbecco’s Modified Eagle’s Medium - high glucose (DMEM, Cat No. D6429-6X500ML), minimum essential medium eagle (MEME, Cat No. M2279-6X500ML), foetal bovine serum heat inactivated (FBS, Cat No F9665-500ML), non-essential amino acid solution (Cat No. M7145-100ML), L-glutamine (Cat No. G7513-100ML), dimethyl sulfoxide (DMSO, Cat No. D2438-50ML), Dulbecco’s phosphate buffered saline, (Cat No. D8662-6x500ML), 0.4% trypan blue solution (Cat No.T8154-100ML), sodium pyruvate (Cat No. S8636-100ML), oleic acid (OA; 99% purity, Cat No. O1008), cis-5,8,11,14,17-eicosapentaenoic acid (EPA; 99% purity, Cat No. E2011-25MG), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA; 98% purity, Cat No. D2534-25MG), penicillin (10,000U/ml) streptomycin (10mg/ml) (Cat No. P4333-100ML), amphotericin B (2.5ng/ml, Cat No. A2942-100ML) and phenol red free trypsin-EDTA solution (1X), (Cat No 59430C-500ML) were all purchased from Sigma (UK). 0.25% trypsin-EDTA solution (1X) and phenol red (Cat No. 25200072-500ML) were purchased from Invitrogen (USA). 15ml centrifugation plastic tubes, 50 ml self-standing centrifugation plastic tubes, T-25, and T-75 culture flasks, 6, 12, and 24 well cell culture cluster and 96-well microplate were all purchased from Corning, (Amsterdam, Netherlands). 25ml plastic tubes, Tissue culture dishes (100/20mm) and serological plastic pipettes 10ml were from Sarstedt (Leicester, UK).
2.2.2. Equipment


2.2.3. Cell thawing

A cryovial was removed from liquid nitrogen and immediately place in water bath at 37°C for 2 min. When about 80% of the cryovial’s content has thawed, the cells were pipetted out into a T75 flask, topped up with 8ml of pre-warmed complete medium and then placed in the incubator. After 24 hours, cells were investigated to insure that they were attached. At the same time the culture medium was changed to remove any non-adherent cells, replenish nutrients, and remove any dimethyl sulfoxide (DMSO) residue.

2.2.4. Cell line maintenance

Cells were maintained in 100% humidified incubator at 37°C with 5% (v/v) CO2. All culturing procedures were carried out in a class II microbiological safety cabinet to provide a sterile cell culturing condition. HaCaT keratinocytes were cultured in DMEM medium supplemented with 10% foetal bovine serum (FBS), 1% of the antibiotic penicillin streptomycin (PS) and 1% of the antifungal amphotericin B. 46BR.1N cells
were cultured in MEME medium supplemented with 15% foetal bovine serum (FBS), 1% of non-essential amino acids, 1:100 of sodium pyruvate (100mM), 1:100 of L-glutamine (200mM), 1% penicillin streptomycin (PS) and 1% amphotericin B. All cells were grown in T-75 culture flasks with vented caps.

Cells were observed every day under a microscope to ensure that they continued to grow. When the cells had reached about 90% confluence they were passaged. The medium was aspirated and 5ml of pre-warmed (37°C) HBSS solution was added. In order to wash the cells, the HBSS solution was gently swirled inside the flask and after it discarded, 4 ml of warm (37°C) 0.25% trypsin-EDTA solution was added and the cells were incubated at 37°C for 15-20 minutes for HaCaT and 2-5 min for 46BR.IN, to detach them from the wall of the flask. Cells were then transferred into 15 ml centrifuge tube. The flask was then rinsed with 4-5 ml appropriate complete cell culture medium and added to the cells in the centrifuge tube to neutralise the trypsin. The tube was spun down (2,000 rpm, 5 min), the supernatant was removed and replaced by 4 ml of fresh complete medium. The cells were then re-suspended by gently pipetting the medium up and down. Then, the cells were counted (section 2.2.8) and an appropriate volume of cell suspension was transferred into new cell culture flasks or cell culture dishes, as needed. Fresh medium was added to cover the surface of the flask (8 ml added to T-75 and 10 ml added to 100/20 mm Petri dish). All flasks and or dishes were then placed in the incubator and used for further work.

2.2.5. Cell treatment with fatty acids

All fatty acids (EPA, DHA and OA) were dissolved in DMSO to prepare stock solutions of 50mM. These solutions were aliquoted to 50µl and stored at -20°C until further use (appendix 1 section 1.1 – 1.3). Cells were treated with either 10µM or 50µM (2µl or 10µl from the 50mM stock solution was added to 100/20 mm Petri dish containing of 10ml of the medium and 70% confluent cells). Control treatment was carried out by adding 10µl DMSO into 10ml of medium. All cells were then incubated for 72 h before UV irradiation.
2.2.6. Cell irradiation

After 72 h incubation period, the medium was removed and the cells were washed with 5ml/dish pre-warmed sterile PBS (37°C) to remove any remaining media and floating cells. Cells were then covered with a small volume (5ml/dish) of pre-warmed sterile PBS before the UVR treatment. The UV lamp was switched on and left for 10 min to stabilize. Light meter was used to measure the irradiance of the lamp (mW/cm²) to make sure that the lamp had stabilized. Typically, this value was at 0.33 mW/cm². The cover of the Petri dish was removed and cells were placed at the base of the UV box, 30 cm under the UV lamp and irradiated for the required duration. The exposure time was calculated as follows: Exposure time (sec) = Dose (mJ/cm²)/UVR intensity (mW/cm²). This corresponds approximately to 45 sec for 15mJ/cm². After irradiation, PBS was aspirated, discarded and replaced with 10ml of serum free media. The cells were further incubated for 24 hours at 37°C with 5% (v/v) CO₂.

2.2.7. Collection of cell pellets and culture media

24 pre-labelled sterile 15 ml centrifuge tubes were prepared before starting the collection of cells pellets and culture media. In each experiment 12 petri dishes were used and each experiment was performed in duplicate (A and B). Experiment generated samples were as follows: Control, Control with UVR, Fatty acid at 10 µM, Fatty acid at 10 µM with UVR, Fatty acid at 50 µM and Fatty acid at 50 µM with UVR. The fatty acids used were either OA or DHA or EPA as indicated in the figure legends.

The culture medium from each dish was collected 24 hours post UVR by a serological pipette and transferred into sterile pre-labelled tube. The medium was stored at -80°C for further use. To collect the cells, HaCaT and 46BR.IN were incubated in 4 ml of warm (37°C) 0.25% trypsin-EDTA solution for 15-20 minutes or 2-5 minutes respectively. Trypsin was neutralised by adding 4 ml of warm complete medium; the content of the dish was then transferred to a pre-labelled 15 ml centrifuge tube. The tube was centrifuged using 2,000 rpm, for 5 min. The supernatant was removed and replaced by 5ml of PBS. The cells were then re-suspended by gently pipetting the PBS up and down.
In order to collect and store the cells; the cell suspension was spun down again at 2,000 rpm, for 5 min at room temperature, then the supernatant was removed and the cell pellet was stored at -80°C to be used for analysis.

2.2.8. Cell counting

In order to count the cells, 100µl of the cell suspension was transferred into an Eppendorf® tube. A dilution 1:1 was done when the cell suspension was mixed with 100µl of 0.4% trypan blue solution. The haemocytometer slide was cleaned using 70% ethanol, the face of the slide was moistened with exhaled breath and a glass coverslip was affixed (Figure 2.1). 10µl of the cell suspension was transferred into the chamber. The cells were counted in 4 grids per chamber (Figure 2.1) under the microscope, using the 10x focus. The average cells of 4 grids were calculated and this number was used to count the total number of cell as follows:

Number of cells/ml = [average cell number] x [dilution factor (2)] x [10^4/ml]. Eq. (1)

Total number of cells = [Cells/ml] x [volume of origin al cell suspension (ml)]. Eq. (2)

2.2.9. Cryogenic storage of the cells

Cells were stored in liquid nitrogen following the procedure described below: 4ml of trypsin-EDTA was added into the culture flask to detach the cells. The detached cells were pelleted by centrifugation at 2,000 rpm, for 5 min. The supernatant was aspirated and the cells re-suspended in 2 ml cryogenic solution consisting of 90% (v/v) FBS and 10% DMSO, 1ml of the cell stock was then transferred into each cryogenic vial (Nunc) and cells were stored in - 80°C for 48 hours as a protective step to minimize damage and death; before they were transferred into liquid nitrogen (-196°C) for long time storage.
Figure 2.1. Side view of the haemocytometer slide. Adapted from (www.MicrobeHunter.com).

Figure 2.1. A. The haemocytometer chamber cell. (A) The arrow in the square on the top right corner indicated how the cells were counted (adapted from Veterinary Hematology and Cytology, SCIE 19207). (B) Showed how the cells were counted at the edge of each square. Adapted from (www.pheculturecollections.org.uk).

2.3. Western blotting

2.3.1 Chemicals

Bromophenol blue (Cat No. B5525), Tween® 20 (Cat No. P1379), phosphate buffered saline tablets (Cat No. P4417), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (Cat No. E5513), glycerol (Cat No. G8773), Sodium dodecyl sulfate (Cat No. 69)
L3771), Trizma® base (Cat No. T4661), acetic acid (Cat No. 34245), glycine (Cat No. G8898), methanol (Cat No. 494437), ammonium persulfate (Cat No. A3678), TEMED (Cat No. T9281), Fast Green FCF (Cat No. F7252), ponceau S solution (Cat No. P7170-1L), protease inhibitor cocktail (PIC) (Cat No. P8340), Coomassie® brilliant blue (Cat No. B0149), Luminol (Cat No. 123072), P-Coumaric acid (Cat No. C9008), GBX fixer (Cat No. P7167 - 5GA), and GBX developer (cat No. P7042 - 5GA) all were obtained from Sigma (UK). Novex® ECL HRP chemiluminescent substrate reagents 2x125 ml (Cat No. WP 20005) was from Invitrogen (UK). AcrylaFLOWGel 30% (Cat No. H17344) and BisacrylaFLOWGel 2% (Cat No. H17356) were supplied by Flowgen Bioscience (UK). DC Protein Assay Reagents (Cat No. 500-0116), lyophilized bovine serum (Cat No. 500-0007), dithiothreitol (DTT) (Cat No. 161-0611), 10x Tris/glycine buffer (Cat No. 161-0734-1L) and 10x Tris/glycine/SDS buffer (Cat No. 161-0772-5L) were from BioRad (USA).

2.3.2. Primary and Secondary Antibodies

COX-2 (human) polyclonal antibody (72KDa), from rabbit (Cat No. 160107), COX-2 (mouse) monoclonal antibody (72KDa), (Cat No. 160112), Fatty Acid Amide Hydrolase polyclonal antibody, from rabbit (63KDa), (Cat No. 101600), NAPE-PLD (6-20) polyclonal antibody (46KDa), from rabbit (Cat No. 10306), Fatty Acid Amide Hydrolase Western Ready Control (Cat No. 10010182) and COX-2 (human) Western Ready Control (Cat No. 10009624) were obtained from Cayman (USA). Anti-biotin, HRP-linked Antibody from goat (Cat No. 7075S) and Biotinylated Protein Ladder Detection (Cat No. 7727S) were from Cell Signaling (USA). Anti-GAPDH (6C5) mouse monoclonal antibody (Cat No. AB8245) and brain: cerebellum (right) (Human) whole cell lysate - adult normal tissue (Cat No. AB30069) were provided by AbCam (UK). ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey) (Cat No. NA934) and ECL Mouse IgG, HRP-Linked Whole Ab (from sheep) (Cat No. NA931) were from GE Healthcare (UK).
2.3.3. Various consumables and small items
Syringes PP/E with removable needle (Cat No. Z116890), Whatman® filter paper (Cat No. Z146382), Parafilm® M (Cat No. P7793), exposure cassette Kodak® BioMax™ (Cat No. C4729) and Kodak® X-Omat LS film - size 5 in. (13 cm) × 7 in. (18 cm) (Cat No. F1274-50EA) were from Sigma (UK). Immobilon Blotting Filter Paper, 7x8.4 cm sheet (Cat No. IBFP0785C) and Immobilon-P Membrane, PVDF, 0.45 µm, 15 x 15 cm sheet (Cat No. IPVH15150) were purchased from Millipore (USA). Mini incubation trays (Cat No. 170-3902) were from BioRad. Cassettes, 1.0 mm (Cat No. NC2010), Combs, 1.0 mm 10 well (Cat No. NC3010), Invitrolon™ PVDF/Filter Paper Sandwiches (Cat No. LC2005) and Xcell SureLock Mini-Cell mark (Cat No. EI0002) were purchased from Invitrogen (USA). Mini-PROTEAN® TGX precast gels 10% and 10-well comb, 50µl/well (Cat No. 456-1034) were from BioRad (USA).

2.4. Sample preparation
2.4.1. Protein extraction
Cell pellets were defrosted in ice and then re-suspended initially in 200µl of ice cold freshly prepared sample buffer (Table 2.1). (Appendix 3 section 3.1.5). 1:100 ice cold protease inhibitor cocktail (PIC), that contained 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin and aprotinin, was then added and the suspension was mixed and kept in ice for 10 minutes. The suspension was homogenized by mixing repeatedly using a 5 ml syringe for 10 times to lyse cellular membranes. If foam was produced during lysis the suspension was centrifuged 3 times to reduce it. The suspension was then incubated on ice for 15 minutes, and then mixed and centrifuged at 13,000 rpm for 5 min. The resulting supernatant that contained the cellular protein was transferred to a new Eppendorf tube and kept on ice waiting for the protein assay to be done or kept in -20°C for further use.
Table 2.1: Composition of the sample buffer used for protein extraction.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration</th>
<th>Final volume or weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>10%</td>
<td>2.5ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.02M</td>
<td>0.19g</td>
</tr>
<tr>
<td>SDS</td>
<td>6%</td>
<td>1.5g</td>
</tr>
<tr>
<td>Tris base</td>
<td>62.4mM</td>
<td>0.19g</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>25ml</td>
</tr>
</tbody>
</table>

2.4.2. Determination of protein concentration

The measurement of the protein concentration was carried out using the BIO RAD DC protein assay kit. This colorimetric assay for the protein concentration is based on the Lowry assay (Lowry et al., 1951). The protein reacts with alkaline copper solution (reagent A) and then reacts with folin reagent (reagent B) and forms a blue colour with maximum absorbance at 750nm and minimum at 450nm.

A calibration line (Figure 2.4) was constructed using six dilutions of bovine serum albumin (BSA) (0.1, 0.2, 0.4, 0.6, 0.8 and 1 (mg/ml)) (appendix 2 section 2.1). As they left for the blank, 5µl of the sample buffer (appendix 3 section 3.1.5) were added into the first three wells of the 96-well microplate (Figure 2.2). Then, 5µl of each BSA dilutions were added in triplicate into the 96-well microplate (Figure 2.3). Three dilutions (1:2, 1:5 and 1:10) were prepared from each test sample. 1ml of reagent A was mixed with 20µl of the surfactant solution (Reagent S) before adding it into every well of a 96-well microplate. Then, 25µl and 200µl of reagent A and B were also added to each well. The microplate was then covered with foil and left for 15 min at room temperature. The absorbance was read at 650nm using a microplate reader, run by the genesis software version 2 (appendix 2 section 2.2). The protein content was calculated based on the equation corresponding to the calibration curve and the concentration was estimated for all test samples (appendix 2 section 2.3).
Figure 2.2: 96-wells microplate (Adapted from www. Edgebio.com)

Figure 2.3: Template showing how the samples were loaded into the 96-wells microplate

Figure 2.4: typical calibration line plotted from BSA standards
2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis is a system used to separate large molecules, for example, proteins or DNA which as charged atoms can be determined in an electric field as indicated by their atomic weight. Protein movement in the gel relies upon its charge and atomic weight. Additionally, protein mobility dependent on the pH of the medium encompassing the protein and on the gel itself. The movement rate of the proteins is also dependent on gel concentration.

![Figure 2.5: Schematic representation of western blotting and detection procedure](Adapted from http://missinglink.ucsf.edu/lm/molecularmethods).

2.5.1 SDS-PAGE: Gel preparation

The lower resolving gel was prepared as described in the Table 2.2; (appendix 3 section 3.1.3). Gel was poured into a disposable gel cassette approximately 1 mm above the first division line. The gel was poured slowly into the cassette to avoid formation of air bubbles. In order to prevent oxygenation of the stacking gel during the polymerization, 300-500 µl of distilled water was added to the top of the gel. Polymerization of the lower separating gel occurred in about 30-45 minutes at room temperature. During this time the upper stacking gel was made (appendix 3 section 3.1.3) (without adding 10%
ammonium persulfate, (APS). When the lower gel polymerization was completed (usually after 30 - 40 minutes), the distilled water was removed and 10% APS was added to the upper stacking gel which applied on top of the solidified resolving gel. 1.5mm well combs were inserted into the gel cast for the formation of loading wells in the gel. The upper stacking gel was then left for about 15-20 min for solidification. SDS-PAGE was performed in 8% gel as described below in Table 2.2.

Table 2.2 Reagents used for the preparation of the acrylamide gel.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating (lower) gel 8%</th>
<th>Stacking (upper) gel 4.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>7.8 ml</td>
<td>2.2 ml</td>
</tr>
<tr>
<td>Bis-Acrylamide</td>
<td>3.12 ml</td>
<td>900 µl</td>
</tr>
<tr>
<td>Gel-Tris buffer</td>
<td>7.5 ml</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>11.6 ml</td>
<td>8.15 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>30 µl</td>
<td>30 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>90 µl</td>
</tr>
</tbody>
</table>

2.5.2 SDS-PAGE: Gel electrophoresis

When preparing the SDS-PAGE, the samples to be analysed were denatured. SDS loading buffer was added to the cell lysates and then boiled to unfolding protein into primary (linear) form (Figure 3.7). SDS is an anionic detergent responsible only for the disruption of non-covalent bonds in the proteins. Therefore the reducing agent; dithiothreitol (DTT) was added to disrupt the disulfide covalent bonds which were not affected by SDS (Figure 3.7). SDS also, confers negative charge in protein samples to allow protein migration during SDS-PAGE. DTT was added at a concentration of 50mM; an equal volume was added to the protein sample in 1:1 dilution. 0.08% of the tracking agent bromophenol blue was then added to the protein sample and the mixture was boiled for 5 min. The molecular weight markers were boiled at the same time for 3 min. A biotinylated protein ladder was used as molecular weight marker on Western blots when using the horseradish peroxidase (HRP). The molecular weight ladders are a mixture of purified proteins covalently coupled to biotin that resolve to 10 bands that have a size range of 9-200 kDa. The electrophoresis chamber was then assembled and the running buffer was made (appendix 3 section 3.1.1). The comb was removed after the gel was polymerized. The gel cassette was placed into the electrophoresis chamber.
the tank was filled with running buffer and the protein samples were loaded into the wells on the gel. Electrophoresis was carried out at a constant voltage of 125V for approximately 90 minutes or until the bromophenol blue stain reached the end of the cassette. The electrophoresis chamber was then assembled and the running buffer was prepared (appendix 3 section 3.1.1). The comb was removed after the gel was polymerized. The gel cassette was placed into the electrophoresis chamber the tank was filled with running buffer and the protein samples were loaded into the wells on the gel.

![Figure 2.6: Protein denaturation using heat and SDS](image.png)

Figure 2.6: Protein denaturation using heat and SDS; circles indicate that the fold caused by disulfide covalent bonds were not affected by heating and SDS. DTT eliminated any tertiary or quaternary structures. Adapted from (www.ruf.rice.edu).

2.5.3. Wet transblotting

The PVDF membrane was activated in 100% methanol for 30 seconds and rinsed with distilled water for about 2 min until hydrophobicity (apparent by beading of water on the surface) disappears. In order to equilibrate the membrane, the membrane was incubated in 15ml of the transblot buffer (appendix 3 section 3.1.2) for about 5 min. When the electrophoresis was completed, the gel cassette was removed from the electrophoresis tank, opened and the upper stacking gel was removed. The lower gel was placed in a blotting module (Figure 2.7) which allowed the protein in the gel to be transferred to the PVDF membrane; the position of the PVDF membrane inside the blotting module is shown in Figure 2.8 A and B. The blotting module was then placed in
the electrophoresis chamber and run at 100 V for 2 hours. When the transblotting finished, the PVDF membrane was placed in a plastic square dish containing fast green stain (appendix 3 section 3.1.8) for about 2 min, after that the stain was poured off and destain was added (appendix 3 section 3.1.7). When green bands were apparent on white background, destain was removed. Next, the membrane was placed in another plastic dish containing PBS and left for 2 min to be washed.

2.5.4. Blocking and Immunoblotting

The membrane was replaced in 5% non-fat milk in PBS for 1 hour at room temperature to reduce non-specific antibody binding on non-target proteins. The milk solution was changed after 30 min. Finally, the membrane was washed with PBS for 3 times and submitted to primary antibody incubation overnight in the cold room under agitation. After the incubation with the primary antibody; the membranes were washed with PBST for 3 time 10 min each and 1 time with PBS for 5 min on a shaker. After that, the membrane was incubated with the secondary antibody for 2 hours at room temperature under agitation; the biotinylated protein ladder was added one hour later. The membrane was then washed again as before to prepare for the protein visualization step.
Figure 2.7: Diagram showing the position of the blotting pads inside the blotting module during the transblotting process (Adapted from Invitrogen user manual).

Figure 2.8: Diagram showing the position of PVDF membrane in the blotting module. A and B show the single and double membrane transblotting process respectively (Adapted from; Invitrogen user manual).
2.5.5. Protein visualization

After the final washing step, the membrane was placed in a square dish filled with PBS. PBS was then poured off; the membrane was dried by soft tissue or filter paper and transferred into another clean square dish. 10 ml of freshly made ECL solution (Appendix 3 section 3.1.9) was added into the clean dish covered with foil and left for 5 min at room temperature allowing the reaction between the ECL and HRP antibody (Figure 2.9). The ECL solution was decanted and the PVDF membrane was removed and dried off any excess moisture using tissue paper with the membrane’s protein side up. Then the membrane was placed with the protein side down onto wrinkle free cling film which was then folded over carefully. The cling film was cut and the membrane was transferred to the exposure cassette with the protein side up and the exposure cassette closed.

The exposure cassette was opened in the dark room; a photographic film was placed on the top of the wrapped membrane; then the exposure cassette was closed and some weights put on it for 5 min. During that time, the developing (Developer 50 ml in 200 ml dH₂O) and fixing (fixer 50 ml in 200 ml dH₂O) solutions were prepared. After exposing the film for the required time the cassette was opened and the film was bended at the top left hand corner. The film was then placed in the developing solution tray until black bands appeared (this was visualized by holding up to the light). Subsequently, the film was put in the H₂O tray with gently agitation for 2-3 min. Then, the film was placed in the fixing solution tray with gently agitation for 2-3 min, it was then rinsed under tap water to remove any residue. Finally, the film was left for 10 min to dry either in the drying cabinet or standing up vertically on the bench.

2.5.6. Membrane stripping and re-probing

The PVDF membrane was incubated in a square petri dish with mild stripping buffer (appendix 4 section 4.1.1) for 10 min at room temperature. The incubation was repeated for another 10 min with fresh stripping buffer and the membrane was then washed with PBS for 10 min 2 times, at room temperature and 2 times with PBS containing 0.05%
Tween 20, for 5 min each. Finally, the membrane was incubated with PBS only for 5 min before starting the blocking step (appendix 4 section 4.2).

![Figure 2.9: schematic summarizing the protein visualization process. Adapted from (www.gelifesciences.com)](image)

2.6. Densitometry analysis

Densitometric analysis of Western blots (Schmidt et al., 1987, Gassmann et al., 2009) was carried out using ImageJ software (Schneider et al., 2012). The intensity of the protein band in each treatment was normalized to that of the corresponding GAPDH protein band under the same conditions. The ratio of the intensity of the protein band divided with the intensity of the GAPDH band in the untreated cells was arbitrarily set to 1. The ratio of the intensity of the protein versus the intensity of the GAPDH band in the remaining samples was calculated accordingly.

2.7. Antibody optimization

2.7.1. COX-2 and GAPDH Antibodies

Two different concentrations of the antibodies against COX-2 and GAPDH were used as shown in Figure 2.10. Cellular extract from the fibroblasts cells 46 BR.1N containing 300 µg protein subjected to SDS-PAGE and Western blot analysis as described above.
The PVDF membrane was then cut into three strips. The membrane was incubated overnight with primary antibodies of COX-2 and GAPDH in the cold room followed by incubation with the secondary antibodies for 2 hours at room temperature. The biotinylated protein ladder was added one hour later. Figure 2.11 shows that the loading control GAPDH (36 kDa) appeared clearly in strip 1 with dilution of 1:10,000 indicating that further dilution was necessary and the exposure time (5 min) had to be reduced. The COX-2 band (72 kDa) at a dilution of 1:200 shown in strip 2 was better than that in strip 3 incubated with the primary antibody at dilution of 1:400.

After several experiments, the optimal dilution for the GAPDH antibody was found to be 1:30,000. Also, good quality of bands was achieved when the imaging time was reduced from 5 min to 30 – 60 sec. However, the optimal dilution of COX-2 antibody was 1:200 and the bands required 3 to 5 min exposure time to be seen clearly. Moreover, all the secondary antibodies used in this study were used at dilution range 1:500 – 1:2000.

Figure 2.10: Optimization of COX-2 and GAPDH antibodies. Amount of protein was 300 µg per gel. (A) Showed GAPDH band after the PVDF membrane was incubated with GAPDH (Mouse) monoclonal antibody at dilution of 1:10,000. (B) Showed COX-2 band after the membrane was incubated with COX-2 (Human) polyclonal antibody at dilution of 1:200. (C) Showed COX-2 band after the membrane was incubated with COX-2 (Human) polyclonal antibody at dilution of 1:400. HRP- (Anti-Mouse) 1:1500, HRP- (Anti-Rabbit) at dilution of 1:1500.
2.7.2. Optimization of 12-Lipoxygenase (murine leukocyte) Polyclonal Antiserum

Cells from the fibroblastic cell lines 46 BR.1N were suspended in the lysis buffer (10% Glycerol, 0.02M EDTA, 62.4mM Tris base, 6% SDS and dH₂O) and the protein was extracted as described in section 2.5.1. The protein obtained content was diluted to 2µg/µl. In order to have 30 µg/well, 15 µl of this solution were loaded into each well. After transblotting (section 2.6.2), the PVDF membrane was blocked and then incubated with the primary antibodies of 12-LOX overnight in the cold room. This was followed by incubation with the secondary antibodies in the next day for 2 hours at room temperature. The biotinylated protein ladder was added one hour later. This experiment indicated that a dilution 1:1000 of 12-LOX antibody was slightly high but still acceptable.

| UVR | - | + | - | + | - | + |
| OA 10µM | - | - | + | + | - | - |
| OA 50µM | - | - | - | - | + | + |

Figure 2.11: Optimization of 12-LOX (murine leukocyte) Polyclonal antiserum.
Dilution = 1:1000. HRP (Anti rabbit) secondary antibody, dilution = 1:1000. 12-LOX expression after OA treatment in 46BR.IN fibroblasts in the presence and absence of UVR. Protein dilution was 30 µg/well.
2.8. Analysis of endocannabinoids by LC-MS/MS

2.8.1. Materials

Acetonitrile (HPLC grade), methanol (HPLC grade), chloroform (HPLC grade) and glacial acetic acid (HPLC grade), all were purchased from, Fisher scientific, Loughborough, UK. 50, 100, 250 and 500 µl glass syringes were purchased from SGE, Australia. Glass tubes and glass Pasteur pipettes were purchased from Fisher, Loughborough, UK. Amber glass vial, 100 µl insert vials, crew caps, and septa all were purchased from Kinesis, Bedfordshire, UK.

2.8.2. Experimental description of LC-MS/MS analysis

Each pellet and medium from HaCaT keratinocytes and 46BR.IN fibroblasts was given a code. The purpose of this was to blind the running of the samples in LC-MS/MS analysis. Pellets and medium represent the same experimental design described in section 2.2.7.

All endocannabinoids were analysed by multiple reaction monitoring (MRM) in the positive ionization mode (ES+). Quantification is based on the construction of calibration lines using commercially available standards and deuterated internal standards (AEA-d8 and 2-AG-d8).

In this experiment the following compounds were analysed: AEA, 2-AG, PEA, ALEA, LEA, OEA, STEA, DHEA, MEA, PDEA, POEA, HEA, DGLEA, DPEA, DEA, NEA, LGEA and 1-AG. Chloroform/methanol 2:1(v/v) mixture (appendix 5 section 5.1) was added into the cell pellets or the medium to extract polar and non-polar compounds. Water was then added to form the bilayer phase, where the lipids in the lower chloroform layer and the non-lipid components in the top methanol/water layer. Chloroform was removed by evaporation under nitrogen (N2) and the lipid residue was then reconstituted in 100µl ethanol (HPLC grade). Finally, the extracts were stored at -20 °C before LC/ESI-MS/MS analysis.
2.8.3. Extraction Protocol

All samples (pellets or culture medium) were defrosted in ice before start. 3 ml of ice cold chloroform (CHCl₃): methanol (MeOH) (2:1v/v all HPLC grade) (appendix 5 section 5.1) was added to the cell pellets and mixed well. However, 2 ml of the treated medium were mixed with 9 ml of ice cold chloroform (CHCl₃): methanol (MeOH). After that, 20ng (20µl x 1ng/µl) of the internal standards anandamide-d8 (AEA-d8) and 40ng (40µl x 1ng/µl) 2-Arachidonoyl glycerol-d8 (2-AG-d8) (appendix 5 section 5.2) were added to each sample (pellet or medium) using a Hamilton glass syringe. The syringe was wiped with a clean tissue in between addition of the standard to the sample and refilling the syringe sample. The samples were vortexed and left on ice for 30 min. During that time the refrigerated centrifuge was switch on to cool down to 4 °C and the centrifuge was set to 5 x 1000 RPM. 0.5 ml of Mill Q water was added to each cell pellets; but not to the medium, to generate the two phase layers. The sample was mixed well and centrifuged for 5 min at 4 °C, 5000 RPM. The bottom organic phase was transferred into a clean glass 12ml vial using a glass Pasteur pipette. After that the organic phase was dried down under nitrogen and the extract reconstituted in 100µl of ethanol (HPLC grade) using a Hamilton glass syringe. The syringe was wiped with a clean tissue in between addition. The ethanol was dripped around the inside of the tube to collect dried lipids. Then, the collection was mixed well and centrifuged for 10 seconds. At that point all the extracts were transferred to an insert in a labelled amber vial with septa and lid. A parafilm was wrapped around the lid to stop evaporation of solvent and stored on ice until finished. The syringe was washed with 3x ethanol washes 1 and 2 and the extracts were kept in -20 °C while awaiting LC-MS/MS analysis.

2.8.4. LC-MS/MS analysis of endocannabinoids and NAE

AEA, 2-AG, PEA, ALEA, LEA, OEA, STEA, DHEA, MEA, PDEA, POEA, HEA, DGLEA, DPEA, DEA, NEA, LGEA and 1-AG were separated using an isocratic system composed of two solvents (A and B) mixed at constant ratio of 30:70 (v/v).
Solvent A was acetonitrile: water: acetic acid, 2:98:0.5 (v/v/v) and solvent B was acetonitrile: water: acetic acid, 98: 2: 0.5 (v/v/v).

The run time was set at 69 min. Separation was performed on a C18 (Luna 5µl, 150 x 2.0 mm) column. The injection volume was 3µl for standards and the biological extracts. Details of MRM transitions, as well as indicative retention times, for 12 endocannabinoids and their congeners were shown in Table 2.3. The instrument is operated in the positive ionisation mode, and for all compounds, the MS/MS settings are as follows: capillary voltage 4500 V, source temperature 100 °C, desolvation temperature 400 °C, cone voltage 35 Dwell time 0.2 s. The transitions should be split at 10 min so that those that are eluted before 10 min and those that are eluted after 10 min have a more focused analysis. LC/ESI-MS/MS analysis was performed on a Waters Alliance 2695 HPLC pump with a Waters 2690 autosampler coupled to an electrospray ionisation (ESI) triple quadruple Quattro Ultimo mass spectrometer. The Mass Lynx™ V 4.0 was used as operating software to control the instrument and data acquisition.

**Table 2.3 multiple reaction monitoring (MRM) transitions for the LC/ESI-MS/MS assay of endocannabinoids and their congeners**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM</th>
<th>Collision energy (eV)</th>
<th>Retention time* (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEA</td>
<td>300&gt;62</td>
<td>13</td>
<td>32.57</td>
</tr>
<tr>
<td>ALEA</td>
<td>322&gt;62</td>
<td>14</td>
<td>20.53</td>
</tr>
<tr>
<td>LEA</td>
<td>324&gt;62</td>
<td>15</td>
<td>25.89</td>
</tr>
<tr>
<td>OEA</td>
<td>326&gt;62</td>
<td>16</td>
<td>38.92</td>
</tr>
<tr>
<td>STEA</td>
<td>328&gt;62</td>
<td>15</td>
<td>51.46</td>
</tr>
<tr>
<td>AEA</td>
<td>348&gt;62</td>
<td>15</td>
<td>26.30</td>
</tr>
<tr>
<td>AEA-d8</td>
<td>356&gt;63</td>
<td>16</td>
<td>25.92</td>
</tr>
<tr>
<td>DHEA</td>
<td>372&gt;62</td>
<td>15</td>
<td>25.51</td>
</tr>
<tr>
<td>2-AG</td>
<td>379&gt;287</td>
<td>18</td>
<td>35.01</td>
</tr>
<tr>
<td>2-AG-d8</td>
<td>387&gt;295</td>
<td>20</td>
<td>34.13</td>
</tr>
<tr>
<td>1-AG</td>
<td>379&gt;287</td>
<td>18</td>
<td>37.63</td>
</tr>
<tr>
<td>MEA</td>
<td>272&gt;62</td>
<td>12</td>
<td>4.76</td>
</tr>
<tr>
<td>PDEA</td>
<td>286&gt;62</td>
<td>12</td>
<td>5.42</td>
</tr>
<tr>
<td>POEA</td>
<td>298&gt;62</td>
<td>14</td>
<td>5.07</td>
</tr>
<tr>
<td>HEA</td>
<td>&gt;62</td>
<td>12</td>
<td>7.27</td>
</tr>
<tr>
<td>DGLEA</td>
<td>350&gt;62</td>
<td>14</td>
<td>6.11</td>
</tr>
<tr>
<td>DPEA</td>
<td>374&gt;62</td>
<td>14</td>
<td>5.79</td>
</tr>
</tbody>
</table>
2.8.5. Standards for quantification and calibration lines

The following standards were used to generate calibration lines: AEA, 2-AG, PEA, ALEA, LEA, OEA, STEA, DHEA, MEA, PDEA, POEA, HEA, DGLEA, DPEA, DEA, NEA, LGEA and 1-AG (appendix 5 section 5.3). These calibration lines were used to calculate the quantity of each one of the endocannabinoid compound of interest. At least 5 concentrations each calibrate should be used for each calibration line and concentrations ranged from 1-200 pg/µl (Table 2.5). However, the range of these calibration lines can be adjusted depending on the amount of compound found in the samples. Standards are made up in amber vials with 100 µl inserts, sealed with Parafilm and stored at -20 ºC until analysis. By integrating the chromatograms from each standard concentration, the area under the peak for each compound from the first and second injections can be determined. Additionally, as each injection also contains an internal standard representative of each type of endocannabinoid, these peak-area values can be normalized as a peak-area to internal standard ratio. The mean peak-area to internal standard ratio for each concentration of standard can be used to construct a calibration line, which can be analyzed by the least-squares linear regression method to calculate its gradient.

Table 2.4: Guidelines for the generation of standards of different concentrations to be used to construct an endocannabinoid calibration line

<table>
<thead>
<tr>
<th></th>
<th>Final concentration (pg/µl)</th>
<th>20</th>
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<td>400 pg/µl cocktail (µl)</td>
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<td>1 ng/µl 2-AG-d8 (µl)</td>
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*Final volume does not include 40 µl of 1 ng/µl 2-AG-d8 as this internal standard is dried down before the addition of other reagents, so it does not contribute to the final volume.
2.9 Clinical study

The clinical study was conducted by the group of Professor LE Rhodes at the Salford Royal Photodermatology Unit. All samples were collected by Sarah Felton (OXFORD UNIVERSITY HOSPITALS NHS TRUST). This work is part of a research collaboration between the two labs.

2.9.1. Experimental design

Prof LE Rhodes and Dr Sarah Felton designed and undertook this study. Sixteen healthy volunteers aged 23-59 years from Greater Manchester, UK, were engaged in this study: 10 white Caucasians of skin phototype II and 6 South Asians of skin phototype V. Subjects were excluded from participation if pregnant or breastfeeding, taking vitamin D supplements or photoactive medication, if they had a personal history of skin cancer, photosensitivity or systemic lupus erythematosus, or if they had used a sunbed/sunbathed in the 3 months prior to commencement of the study, or during the study period. All received and read the study information leaflets at least a week prior to their decision to take part. They subsequently attended for an initial assessment visit where the study was discussed and they were given the opportunity to ask questions before providing their consent to participate. The Tables (2.5 and 2.6) below illustrates the time points of UV radiation and blood samples collection throughout the study. Volunteers were given short, sub-erythemal exposures to UV radiation three times weekly for 6 weeks, whilst wearing informal summer clothing.
Table 2.5. Schematic pattern showing the multiple time points of UVR exposure and blood sample (BS) collection days

Table 2.6. Sample code: The highlighted days showing the days of UVR exposure and/or blood sample collection

2.9.2. Simulated summer sunlight exposures

A Philips HB588 irradiation cabinet (Figure 2.13) was used to deliver whole body UV exposure. This cabinet was fitted with fluorescent tubes in an alternating pattern, to provide an UV emission spectrum as close as possible to summer sunlight (95% UVA: 320–400 nm, 5% UVB: 290–320 nm). Emission from the radiation cabinet was characterised using a Bentham DTM300 spectroradiometer and monitored using an Ocean Optics S2000 spectroradiometer by Dr R. Kift (School of Earth, Atmospheric and Environmental Sciences, University of Manchester).
2.9.3. Irradiation Protocol

Wearing protective eye goggles, standardised T-shirts and knee-length shorts, volunteers lay prone (i.e. face down) on the sunbed with the canopy closed. Approximately 35% of their skin surface area was exposed. A 6-week course of exposures was selected to be concordant with the length of the summer school holiday period when the population is most exposed to sunlight. The course of simulated summer sunlight was given three times a week in January and February when ambient UVB is negligible at UK latitudes and people have their lowest vitamin D status (Webb and Engelsen, 2006). An exposure of 1.35 standard erythemal dose (SED) (Diffey et al., 1997) was given to each subject at every visit. The time required to deliver this dose was found to be 6.5 min after accurate measurement of cabinet UV irradiance (Taylor et al., 2002); a constant UV dose was maintained throughout the study by adjusting for any decrease in irradiance by increasing delivery time.

2.9.4. Skin colour measurements

All participants had assessments of their buttock skin colour non-invasively at baseline (i.e. prior to sunbed exposure) using a Minolta CM-2500d hand-held spectrophotometer (Figure 2.14). Readings were taken when the volunteers had been placed prone for 3 min prior to their irradiation, to eliminate heat from the sunbed and postural variations.
in circulation as confounding factors. UV-exposed and UV-protected buttock skin subsequently underwent weekly spectrophotometric measurements. Data were recorded in standard three-dimensional L*A*B* format, as recommended by the Commission International de le'Eclairage (Robertson, 1978). L* represents white-black differentiation, whereby an L* of 100 is pure white and conversely L* of 0 is pure black, A* values reflect the balance between green (negative) and increasing redness (positive) whilst B* represents the differentiation between blue (negative) and yellow (positive). The spectrophotometer was ‘Zero calibrated’ at study commencement by Dr D Allan, in addition to being calibrated using its ‘White Calibration’ system each time the power was turned on prior to each participant’s measurements, to guarantee accuracy of readings. Readings were made in triplicate at each site and the mean calculated.


2.9.5. Blood collection and serum separation

Each volunteer had a venous blood sample taken at baseline for measurement of endocannabinoids (anandamide and 2-arachidonoyl glycerol). Samples were repeated at the beginning of each week of the study. Samples were centrifuged in a centrifuge at 2400rpm for 15 minutes and once the serum had separated it was aspirated using a pipette and stored in a glass sample vial in a -20°C freezer. The samples were then transferred to the University of Manchester on dry ice and stored at -80°C awaiting LC-MS/MS analysis.
2.9.6. Lipid Extraction

The total number was 153 serum samples, 147 samples had the same volume (400µl), and the other 16 samples were in the range from 350µl to 1000µl. All the samples were defrosted in ice. 3 ml of ice cold chloroform (CHCl3): methanol (MeOH) (2:1 v/v all HPLC grade) (appendix 5 section 5.1) was added to the serum and mixed well. After that, the whole volume of each sample was transferred into a new 12 ml glass tube. 20ng (20µl x 1ng/µl), 20µl of the internal standards Anandamide-d8 (AEA-d8) and 40ng (40µl x 1ng/µl), 40µl of 2-Arachidonoyl glycerol-d8 (2-AG-d8) (appendix 5 section 5.2) was added to each sample using a Hamilton glass syringe. The syringe was wiped with a clean tissue in between addition of the standard to the sample and refilling the syringe sample. The samples were mixed well and left on ice for 30 min (Samples were mixed each 10 min). During that time the refrigerated centrifuge was switched on to cool down to 4 °C and the centrifuge was set to 5 x 1000 RPM. 0.5 ml or 1 ml of Mill Q water was added to each sample, to generate the two phase layers. The samples were mixed well and centrifuged for 5 min at 4 °C, 5000 RPM. The bottom organic phase was transferred into a clean glass 12ml vial using a glass Pasteur pipette. After that the organic phase was dried down under nitrogen and the extract reconstituted in 100µl of ethanol (HPLC grade) using a Hamilton glass syringe. The syringe was wiped with a clean tissue in between addition. The ethanol was dripped around the inside of the tube to collect dried lipids. Then, the collection was mixed well and centrifuged for 10 seconds. At that point all the extracts were transferred to an insert in a labelled amber vial with septa and lid. A parafilm was wrapped around the lid to stop evaporation of solvent and stored on ice until finished. The syringe was washed with 3x ethanol washes 1 and 2 and the extracts were kept in -20 °C awaiting for LC-MS/MS analysis as described in sections 2.8.4 and 2.8.5.
2.10. Statistical Analysis

Data are presented as mean ± standard deviation where appropriate. In order to determine that our data are parametric or non-parametric, all the data were subjected to Shapiro-wik’s test (P>0.05) and their skewness and standard error were measured. The histogram of all the data was inspected visually. Regarding to the Z value (which was calculated manually by dividing the skewness value on the standard error), some of our data have a small skew but it does not differ significantly from normality. Therefore, it was assumed that the data are approximately normally distributed. Comparisons between groups in chapters 3, 4 and 5 were made with one-way analysis of variance (ANOVA) followed by Tukey post hoc test. In chapter 5 the comparison was carried out between the different time points of UVR exposure in each skin phototype separately. All statistical analyses were done using SPSS Statistics software version 22. P < 0.05 was considered to be a significant level of difference.
CHAPTER 3: The effect of n-3 PUFA on the formation of endocannabinoids and N-acylethanolamines in HaCaT keratinocytes and 46BR.IN fibroblasts in response to ultraviolet radiation

3.1 Introduction

The endocannabinoid system has been identified in different types of skin cells and tissues as reported in several studies (Mechoulam et al., 1998; Howlett et al., 2002; Pacher et al., 2006; Di Marzo, 2008). These studies reported the existence of AEA and 2-AG, in human skin along with the enzymes that are involved in their synthesis and metabolism (Calignano et al., 1998; Berdyshev et al., 2000; Maccarrone et al., 2003; Karsak et al., 2007). Moreover, the cannabinoid receptors CB1 and CB2 were identified in many types of human and murine skin cells such as organ-cultured hair follicles, SZ95 sebocytes, cutaneous nerve fibres, mast cells and epidermal keratinocytes (Ständer et al., 2005, Ibrahim et al., 2005, Blázquez et al., 2006, Telek et al., 2007b, Telek et al., 2007a, Casanova et al., 2003). Endocannabinoids are also involved in the regulation of human epidermal homeostasis. AEA, for instance has been found to inhibit the differentiation of cultured normal human epidermal keratinocytes (NHEKs) and HaCaT keratinocytes (Maccarrone et al., 2003; Paradisi et al., 2008). Endocannabinoids also exert a protective role in skin allergic inflammation (Karsak et al., 2007).

The endocannabinoids AEA is an N-acylethanolamines (NAE). Other NAE, such as N-palmitoylethanolamide (PEA), N-oleylethanolamide (OEA) and N-stearoylethanolamide (SEA), show different receptor preference, including affinity for GPR55, GPR18, GPR119, TRPV1 (transient receptor potential channel type V1) or PPARα, while often showing less or no affinity for CB1 or CB2 receptors (Alexander and Kendall, 2007; Di Marzo et al., 2007; Farrell and Merkler, 2008; Hansen and Diep, 2009). Therefore, they were not always classified as endocannabinoids because they lack the affinity to the endocannabinoid receptors. The group of NAE contains more than 80 different conjugates of long-chain fatty acids with amino acids (lipoamino acids; elmaric acids) or neurotransmitters (Burstein and Zurier, 2009; Connor et al., 2010; Tan et al., 2010). NAE can be rapidly synthesized in membranes, released on demand by the same biosynthesis pathway of AEA and degraded by the enzyme FAAH (Bisogno,
Ligresti et al., 2005, Bisogno 2008). In addition, NAE can also be produced through the direct conversion of the n-3 PUFA to yield for example palmitoylethanolamide (PEA) which has anti-inflammatory properties (Klein, Newton et al. 2003, Klein 2005, O’Sullivan 2007, Hoareau, Buyse et al. 2009). Based on all this information we believe that n-3 PUFA have a role in the endocannabinoids biosynthesis. Furthermore, n-3 PUFA, especially EPA and DHA, are considered a dietary component with a promising photoprotective action against the deleterious effects of UVR (Black et al., 1992, Rhodes et al., 1994, Rhodes et al., 1995). They exert their anti-inflammatory effects by their competition with AA as substrates for COXs and LOXs, resulting in the formation of less active prostaglandins and leukotrienes (Lands et al., 1992). N-3 PUFA also reduces UVB-erythemal sensitivity in humans (Rhodes et al., 1994), by inhibition of UVB-induced PGE2 levels in the skin (Rhodes et al., 1995).

3.2 Aims of the study
The aim of this study was to explore the hypothesis that EPA and DHA may play a role in protecting human skin cells against the harmful effects of UVR through their effect on the formation of endocannabinoids and other NAE. This study was carried out using a model system including HaCaT keratinocytes and 46BR.1N fibroblasts. The specific objective was to investigate the effect of n-3 PUFA on the formation of endocannabinoids and other NAE in HaCaT and 46BR.1N cells pellets (intracellular) and cell culture medium (extracellular) in response to UVR.
3.3 Materials and Methods

3.3.1. Material (refer to section 2.8.1 chapter 2)

3.3.2. Experimental description of LC-MS/MS analysis (refer to section 2.8.2 chapter 2)

3.3.3. Extraction protocol (refer to section 2.8.3 chapter 2)

3.3.4. LC-MS/MS analysis of endocannabinoids and NAE (refer to section 2.8.4 chapter 2)

3.3.5. Standards for quantification and calibration lines (refer to section 2.8.5 chapter 2)

3.3.6. Statistical analysis (refer to section 2.10. chapter 2)

3.4. RESULTS

The concentration of fatty acids and UVR dose used for this work were not toxic to the cells. This was established by another research worker in the lab and the data is included in his PhD thesis (Al-Aasswad 2013).

3.4.1. Quantification of endocannabinoids and NAE in irradiated and non-irradiated HaCaT keratinocytes treated with OA

Treatment with OA 10µM or 50µM decreased STEA levels to be 369 pg/mg and 289 pg/mg respectively, in concentration dependent manner compared to the non-irradiated control, 422 pg/mg (Figure 3.1A, compare bar 1 to bars 3 and 5). When these cells were submitted to UV radiation, their STEA levels were 393 pg/mg and 273 pg/mg respectively in OA 10µM and 50µM (Figure 3.1, compare bar 2 to bars 4 and 6). This result indicated that UVR could not reverse OA effect on this mediator. However, STEA level was increased in the irradiated HaCaT control cells, 522 pg/mg, compared to the nonirradiated HaCaT cells (Figure 3.1, compare bar 1 with bar 2).
PEA levels estimated in the untreated cells were 1027 pg/mg (Figure 3.1B, bar 1). Small reduction (836 pg/mg and 900 pg/mg) of the PEA levels was evident in the cells treated with either OA 10µM or 50µM, respectively (Figure 3.1B, compare bar 1 to bars 3 and 5). The highest level of PEA was found in the irradiated control sample, 1374 pg/mg (Figure 3.1, compare bar 2 to bar 1). Submitted OA treated cells 10µM or 50µM to UVR resulted in a dose dependent decrease in PEA levels 982 pg/mg and 827 pg/mg compared to the irradiated control cells (Figure 3.1B, compare bar 2 to bars 4 and 6).

The OEA levels in the control untreated cells were assessed to be 1471 pg/mg (Figure 3.1C, bar 1). This level decreased in a dose dependent manner to 1140 pg/mg and 1099 pg/mg in HaCaT cells treated with OA 10µM or 50µM respectively (Figure 3.1C, bar 3 and bar 5). Exposing the OA treated cells (10µM and 50µM) to UVR also decreased the OEA level in a dose dependent fashion, 1240 pg/mg and 1113 pg/mg compared to the irradiated control cells 1632 pg/mg (Figure 3.1C, compare bar 2 to bars 4 and 6).

Treatment with OA 10µM or 50µM did not induce significant changes in LEA levels, 51 pg/mg and 43 pg/mg compared to the untreated control cells 59 pg/mg (Figure 3.1D, compare bar 1 to bars 3 and 5). In the irradiated cells LEA level was estimated to be 58 pg/mg indicating that UVR did not affect the LEA levels in these cells compared to the nonirradiated control cells (Figure 3.1D, compare bar 1 to bar 2). UV radiation of OA 10µM or 50µM treated cells induced fluctuated levels of LEA, 64 pg/mg and 39 pg/mg respectively, compared to the irradiated control cells 58 pg/mg (Figure 3.1D, compare bar 2 to bar 4 and 6).

DHEA levels in HaCaT cells were estimated to be 480 pg/mg (Figure 3.1E, bar 1). Treatment of HaCaT cells with 10µM OA alone induced a minor increase in the DHEA level 559 pg/mg compared to the untreated cells (Figure 3.1E, compare bar 1 to bar 3) whereas increase of the OA dose to 50µM decreased the DHEA level 265 pg/mg compared to those observed in the untreated cells (Figure 3.1E, compare bar 1 to bar 5). The highest DHEA level was found in the irradiated control sample, which was estimated to be 643 pg/mg (Figure 3.1E, bar 2). This level decreased in a dose
dependent fashion to 469 pg/mg and 226 pg/mg when the OA 10µM or 50µM treated cells were irradiated (Figure 3.1E, compare bar 2 to bars 4 and 6).

AEA levels were estimated to be 433 pg/mg in the control untreated HaCaT cells (Figure 3.1F, bar 1). Treatment with OA 10µM decreased the AEA level to 295 pg/mg (Figure 3.1F, compare bar 1 to bar 3). In addition, further reduction in AEA level 195 pg/mg was seen when the OA concentration was increased to 50µM (Figure 3.1F, compare bar 1 to bar 5). In the irradiated control HaCaT cells AEA level reduced to 371 pg/mg compared to the nonirradiated control cells (Figure 3.1F, compare bar 1 to bar 2). Exposed OA 10µM and 50µM treated cells to UV light dose dependently decreased the AEA level to 263 pg/mg and 173pg/mg (Figure 3.1F, compare bar 2 to bars 4 and 6). This indicated that the OA and UVR synergistically down-regulated AEA levels.

2-AG level was recorded to be 2732 pg/mg in the untreated HaCaT control cells. (Figure 3.1G bar, 1). Concentration dependent decrease, 2255 pg/mg and 1668 pg/mg was observed in 2-AG levels when HaCaT cells were treated with OA 10µM or 50µM (Figure 3.1G compare bar 1 to bars 3 and 5). In the irradiated HaCaT control cells 2-AG level was slightly reduced to 2520 pg/mg compared to the nonirradiated control cells (Figure 3.1G compare bar 1 to bar 2). Minor increase in 2-AG level was seen in HaCaT irradiated cells treated with OA 10µM (Figure 3.1G compare bar 2 to bar 4). However, this level was decreased again when these cells were treated with OA 50µM (Figure 3.1G compare bar 2 to bar 6).

1-AG level was slightly lower in the untreated control HaCaT cells 2208pg/mg compared to the irradiated control cells 2504 pg/mg (Figure 3.1H compare bar 1 and bar 2). The level of 1-AG was also decreased in a dose dependent manner, 2040 pg/mg and 1550 pg/mg respectively in OA 10µM or 50µM treated cells compared to the untreated (Figure 3.1 compare bar 1 to bars 3 and 5). Similar results were observed when OA 10µM or 50µM (2487pg/mg and 1504pg/mg) treated cells were irradiated compared to the irradiated control (Figure 3.1 compare bar 2 to bars 4 and 6).
Figure 3.1 Effect of OA treatment on endocannabinoids and NAE levels in HaCaT keratinocytes in response to UV radiation. As described in Material and Methods, cells were treated either with 10 or 50 µM OA for 72h and then they were irradiated with 15J/cm² UVB. Cells were harvested with 0.25% trypsin-EDTA solution 24h post UVR. Quantification of endocannabinoids and NAE in HaCaT keratinocytes was carried out using LC-MS/MS analysis. Data shown as mean of n = 2 experiments. No statistical analysis was performed.
3.4.2. Quantification of endocannabinoids and NAE in irradiated and non-irradiated HaCaT keratinocytes treated with DHA

Pellets from three independent experiments were extracted as described in materials and Methods (Section 2.7.3) and analyzed for endocannabinoids and N-acylethanolamines levels in irradiated and non-irradiated HaCaT Keratinocyte cells treated with DHA.

The STEA level was estimated to be 483pg/mg in the untreated control HaCaT cells. Treatment with DHA 10µM or 50µM for 72h induced dose dependent small reduction, 473pg/mg and 455pg/mg in the STEA levels compared to the untreated cells (Figure 3.2A compare bar 1 to bars 3 and 5). Compared to the irradiated control cells, STEA level was slightly increased respectively to 505pg/mg and 492pg/mg in cells treated with DHA 10µM or 50µM plus UVR (Figure 3.2A compare bar 2 with bars 4 and 6). No significant differences were seen between groups in this experiment.

Figure 3.2B presented that the treatment with DHA either 10µM or 50µM moderately increased the PEA levels in a dose dependent manner, 2350pg/mg and 2402pg/mg compared to the untreated control cells which showed PEA level at1926pg/mg (Figure 3.2B compare bar 1 to bars 3 and 5). UVR-treated control showed the lowest PEA level in this experiment (1476pg/mg) (Figure 3.2B bar 2). Compared to the irradiated control cells, PEA levels also increased respectively to 2032pg/mg and 1776pg/mg in HaCaT cells treated with DHA10µM or 50µM and submitted to UV radiation (Figure 3.10 compare bar 2 to bars 4 and 6). Statistically, there was a significant difference between DHA 50µM and DHA 50µM plus UV radiation ($P = 0.035$).

OEA level was assessed to be 4030pg/mg in the untreated control cells (Figure 3.2C, bar 1). Treatment with DHA 10µM or 50µM for 72h significantly decreased the OEA levels, in a dose dependent fashion, respectively to 2504pg/mg ($P = 0.001$) and 2460pg/mg ($P = 0.001$) compared to the untreated control cells (Figure 3.2C compare bar 1 to 3 and 5). Likewise, 2 fold decreases was seen in OEA level in the irradiated control cells (1855pg/mg), compared to the non-irradiated control HaCaT cells (Figure 3.2C compare bar 2 to bar 1). Moreover, OEA levels were insignificantly decreased
when the irradiated cells were treated with DHA 50µM, 1794pg/mg (Figure 3.2C, bar 6). However, this level was slightly increased, 2261pg/mg when the irradiated cells were treated with DHA 10µM (Figure 3.2C, bar 4).

LEA levels in the nonirradiated control cells were assessed to be 310pg/mg (Figure 3.2D bar 1) while, after the UVR stimulation of control cells, this level significantly decreased 2 fold to 190 pg/mg ($P = 0.002$) (Figure 3.2D bar 2). Treatment with DHA either 10µM or 50µM for 72h also significantly decreased the LEA levels to 79pg/mg ($P = 0.001$) and 90pg/mg ($P = 0.001$) respectively, compared to the untreated HaCaT cells (Figure 3.2D compare bar 1 to bars 3 and 5). Similar effect was seen, 69pg/mg ($P = 0.002$) and 94pg/mg ($P = 0.01$) when the DHA 10µM or 50µM treated cells were irradiated compared to the irradiated control cells (Figure 3.2D compare bar 2 to bars 4 and 6).

DHEA levels in untreated HaCaT control cells were estimated to be 676pg/mg (Figure 3.2E, bar 1). DHEA level was slightly higher in the irradiated control 704pg/mg, compared to the nonirradiated control cells (Figure 3.2E compare bar 1 to bar 2). DHEA level significantly increased in nonirradiated HaCaT cells treated with DHA 50µM 2045pg/mg ($P = 0.001$), compared to control cells (Figure 3.2E compare bar 1 to bar 5). While, DHA 10µM induced only minor increase in DHEA level, 912pg/mg compared to its control cells (Figure 3.2E compare bar 1 to bar 5). However, DHEA level was significantly increased when HaCaT cells treated with DHA 50µM, 2045pg/mg, compared to those treated with 10µM, 912pg/mg ($P = 0.001$) (Figure 3.2E compare bar 3 to bar 5). In cells treated with DHA 50µM plus UVR DHEA level was also significantly increased to 2382pg/mg compared to the irradiated cells treated with 10µM, 1340pg/mg and to the irradiated control cells as well ($P = 0.001$) (Figure 3.2E compare bar 4 to bar 6 and compare bar 2 to bar 6).

AEA levels in untreated HaCaT cells were estimated to be 321pg/mg, whereas, this level was slightly increased after UV radiation in control cells to be 353pg/mg (Figure 3.2 F, bar 1 and 2). AEA levels was dose dependently increased when HaCaT cells were treated with DHA 10µM or 50µM to 397pg/mg and 421pg/mg respectively (Figure
3.2 F compare bar 3 to bar 5). Similar effect was seen when these treated cells were
irradiated (DHA10µM or 50µM, 413pg/mg and 447pg/mg respectively) (Figure 3.2 F
compare bar 4 to bar 6). However significant differences were only observed between
50µM and irradiated and nonirradiated control cells ($P = 0.03$ and 0.02 respectively)
(Figure 3.2 F compare bar 1 to bar 5 and compare bar 2 to 6).

2-AG level in the control sample was 4827 pg/mg, whereas, its level was almost the
same; 4836pg/mg after UVR exposure (Figure 3.2 G bar 1 and bar 2). Treatment with
DHA 10µM or 50µM for 72h, decreased the 2-AG level respectively to 3822pg/mg and
3651pg/mg, compared to the untreated control cells (Figure 3.2 G compare bar 1 to
bars 3 and 5). Furthermore, 2-AG levels in UVR-treated cells combined with DHA either
10µM or 50µM were also reduced to 3600pg/mg and 4277pg/mg respectively,
compared to the irradiated control cells (Figure 3.2 G compare bar 2 to bars 4 and 6).

1-AG level was estimated to be 5133 pg/mg in the untreated control cells (Figure 3.2 H
bar 1) No major change was seen in 1-AG level 5345pg/mg, after UV irradiation (Figure
3.2 H compare bar 1 to bar 2). Whereas, treatment with DHA 10µM or 50µM for 72h,
significantly decreased 1-AG levels to 3341pg/mg ($P = 0.01$) and 2782pg/mg ($P =
0.002$), compared to the untreated control cells (Figure 3.2 H compare bar 1 to 3 and
5). Additionally, 1-AG level was also significantly decreased in a dose dependent
manner, 3700pg/mg ($P = 0.02$) and 2692pg/mg ($P = 0.001$) when the UVR-stimulated
cells were treated with DHA 10µM or 50µM compared to the irradiated control cells
(Figure 3.2 H compare bar 2 to bars 4 and 6).
Figure 3.2 Effect of DHA treatment on endocannabinoids and NAE levels in HaCaT keratinocytes in response to UV radiation. As described in Material and Methods, cells were treated either with DHA 10 or 50 µM for 72h and then they were irradiated with 15 J/cm² UVB. Cells were harvested with 0.25% trypsin-EDTA solution 24h post UVR. Quantification of endocannabinoids and NAE in HaCaT keratinocytes was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control. Figure B, * = compare to non-irradiated cells treated with DHA 50 µM, §. Figure E, § = compared to non-irradiated cells treated with DHA 10 µM while, ≠ = compared to irradiated cells treated with DHA 10 µM.
3.4.3. Quantification of endocannabinoids and NAE in irradiated and non-irradiated HaCaT keratinocytes treated with EPA

Pellets from three independent experiments were extracted as described in Materials and Methods (Section 2.7.3) and analyzed for endocannabinoids and N-acylethanolamines levels in irradiated and non-irradiated HaCaT keratinocyte cells treated with EPA. Data are presented as mean ± standard deviation where appropriate. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Tukey test using SPSS Statistics software. P < 0.05 was considered as significant level of difference.

STEA levels in HaCaT control cells were assessed to be 389pg/mg (Figure 3.3 A bar 1). Treatment with EPA 10µM or 50µM for 72h did not induce significant changes in STEA levels which was estimated between 382pg/mg and 438pg/mg compared to the untreated control cells (Figure 3.3 A, compare bar 1 to 3 and 5). However, UVR induced minor decreases in STEA level either in control cells or in the treated cells (316pg/mg, 347pg/mg and 357pg/mg compare bar 2 with bars 4 and 6 respectively).

PEA level was calculated to be 2699 in untreated control cells (Figure 3.3 B, bar 1). Treatment with EPA 10 µM or 50 µM resulted in small decreases in PEA levels in these cells, 2162pg/mg and 2288pg/mg respectively compared to untreated control cells (Figure 3.3 B, compare bar 1 to bars 3 and 5). However, PEA level was significantly reduced in irradiated cells to 1935pg/mg compared to nonirradiated HaCaT cells (P = 0.035) (Figure 3.3 B, compare bar 1 to bar 2). Whereas, when the EPA treated cells were subjected to UVR there were no significant changes in PEA levels (1953pg/mg and 2001pg/mg) compared to irradiated control cells (Figure 3.3 B, compare bar 2 to bars 4 and 6).

Untreated HaCaT cells showed OEA level at 2069 pg/mg (Figure 3.3 C, bar 1). Treatment of the HaCaT Keratinocyte cell with two different concentrations of EPA (10µM or 50µM) for 72h increased the OEA levels to 2184 pg/mg and 2564 pg/mg, respectively in dose dependent manner (Figure 3.3 C compare bar 1 to 3 and 5). Likewise, OEA level was also increased in irradiated control cells to 2429 pg/mg.
However, this effect of UVR was reversed in dose dependent way in EPA10µM or 50µM treated cells to 2085 pg/mg and 1807pg/mg, respectively) when they were irradiated (Figure 3.3 C, compare bar 2 to bars 4 and 6).

LEA levels in HaCaT control cells were assessed to be 334 pg/mg (Figure 3.3 D, bar 1) while, after the UVR exposure this level significantly fallen to 158 pg/mg ($P = 0.001$) (Figure 3.3 D, bar 2). Treatment with EPA 10µM or 50µM for 72h decreased the LEA levels to 242 pg/mg and 214 pg/mg, respectively, in dose dependent manner (Figure 3.3 D compare bar 1 to 3 and 5). However, the difference was only significant ($P = 0.012$) between untreated control and EPA 50 µM treated cells (Figure 3.3 D, compare bar 1 with bar 5). In irradiated control cells LEA level was dropped to 158 pg/mg (Figure 3.3 D, bar 2), but raised in EPA10µM treated cells to 198pg/mg (Figure 3.3 D, bar 4) and then decreased further to 120 pg/mg in the EPA 50µM treated cells. (Figure 3.3 D, bar 6).

DHEA levels in the untreated control cells were found to be 1061pg/mg (Figure 3.3 E, bar 1). Treatment with EPA either 10 µM or 50 µM significantly reduced DHEA levels to 783 pg/mg and 754 pg/mg respectively in dose dependent manner compared to the untreated control cells ($P = 0.001$)(Figure 3.3 E, compare bar 1 to bars 3 and 5). Moreover, UVR induced significant down-regulation in DHEA level to 821pg/mg compared to the non-irradiated control cells ($P = 0.001$) (Figure 3.3 E, compare bar 2 to bar 1). These effect also continued when EPA 10µM (733 pg/mg) or 50µM (684 pg/mg) treated HaCaT cells were subjected to UV radiation compared to the irradiated control cells (Figure 3.3 E, compare bar 2 to bars 4 and 6).

Figure 3.3 F showed that treatment with EPA at two different concentrations; 10 µM or 50 µM did not induce any noteworthy changes in AEA levels which was estimated to be 93 pg/mg and 112 pg/mg respectively, compared to untreated control cells, 107pg/mg (Figure 3.3 F, compare bar 1 to bars 3 and 5). However, AEA level was increased in irradiated HaCaT cells treated with EPA 10µM or 50µM to 116pg/mg and 119pg/mg compared to the irradiated control cells that its AEA level was estimated to be 95pg/mg (Figure 3.3 F, compare bar 2 to bars 4 and 6). Statistical analysis showed
significant difference only between irradiated cells treated with EPA 50µM and irradiated control cells ($P = 0.037$).

2-AG levels in the untreated control cells were estimated to be 4099pg/mg (Figure 3.3 G, bar 1). Treatment with EPA 10µM or 50µM for 72h, dose dependently decreased 2-AG levels to 4002pg/mg and 3412pg/mg, compared to the untreated control cells (Figure 3.3 G, compare bar 1 to bars 3 and 5). Similar effect was also observed in the irradiated cells treated with EPA 10µM or 50µM (3091pg/mg and 3023pg/mg) compared to the irradiated control cells (3132pg/mg) (Figure 3.3 G, compare bar 2 to bars 4 and 6).

1-AG levels in the untreated control cells were assessed to be 3967pg/mg (Figure 3.3 H, bar 1), whereas, UV radiation significantly down-regulated 1-AG level to 2481pg/mg ($P = 0.003$) (Figure 3.3 H, bar 2). Similarly, treatment with EPA 50µM significantly decreased 1-AG level to 2359pg/mg compared to the untreated control HaCaT cells ($P = 0.002$) (Figure 3.3 H, compare bar 1 to bar 5). Treatment with EPA 10µM also decreased 1-AG level to 3133pg/mg (Figure 3.3 H, compare bar 1 to bar 3) compared to the untreated control cells. Although, there was decrease effect after UV radiation in EPA 10µM or 50µM treated cells, no significant differences were noted in 1-AG levels (2247pg/mg and 3089pg/mg, respectively) (Figure 3.3 H, compare bar 2 to bars 4 and 6).
Figure 3.3 Effect of EPA treatment on endocannabinoids and NAE levels in HaCaT keratinocytes in response to UV radiation. As described in Material and Methods, cells were treated either with EPA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Cells were harvested with 0.25% trypsin-EDTA solution 24h post UVR. Quantification of endocannabinoids and NAE in HaCaT keratinocytes was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
3.4.4. Quantification of endocannabinoids and NAE in culture medium from irradiated and non-irradiated HaCaT keratinocytes treated with OA

Supernatant from HaCaT Keratinocyte cells treated with OA was analysed for the OEA level. Figure 3.4 A, showed that the OEA level in the untreated medium was assessed to be 369pg/ml (Figure 3.4 A, bar 1). Treatment with OA 10µM and 50µM decreased this level to 295pg/ml and 318pg/ml respectively compared to untreated control medium (Figure 3.4 A, bar 1 to bars 3 and 5). Radiation of control medium up-regulated OEA level up to 421pg/ml compared to the nonirradiated control medium (Figure 3.4 A, bar 1 to bar 2). However, this level decreased to 308pg/ml and 373pg/ml respectively when OA 10µM or 50µM treated HaCaT culture medium were irradiated (Figure 3.4 A, bar 2 to bars 4 and 6).

In the supernatant of untreated control cells STEA levels were found to be 2501pg/ml (Figure 3.4 B, bar 1). Treatment with 10µM or 50µM of OA for 72h did not induce major changes in STEA levels (2501pg/ml and 2250pg/ml) compared to the untreated control culture medium (Figure 3.4 B, bar 1 to bars 3 and 5). On the other hand, UVR increased the STEA level to 3501pg/ml in the irradiated control culture medium compared to the nonirradiated control culture medium (Figure 3.4 B, compare bar 1 to bar 2). This level was similar in the irradiated culture medium treated with OA 10µM or 50µM (3501pg/ml and 3751pg/ml) compare to irradiated control culture medium (Figure 3.4 B, compare bar 2 to bars 4 and 6).

In cell cultural medium of untreated control cells DHEA level was estimated to be 152pg/ml (Figure 3.4 C, bar 1), whereas, there was no much difference in DHEA level in irradiated control culture medium (165pg/ml) compared to the non-irradiated control (Figure 3.4 C, compare bar 1 to bar 2). In addition, treatment with OA either 10 µM or 50 µM did not change DHEA levels (156pg/ml and 154pg/ml, respectively) compared to the untreated control culture medium (Figure 3.4 C, compare bar 1 to bars 3 and 5). However, a small dose dependent decrease was observed in DHEA levels when OA 10µM or 50µM (148pg/ml and 141pg/ml) treated culture medium were irradiated compared to the irradiated control culture medium (Figure 3.27 compare bar 2 to bars 4 and 6).
2-AG levels in the supernatant of the untreated control cells were assessed to be 1193pg/ml (Figure 3.4 D, bar 1). After UVR stimulation, 2-AG level was almost the same as its level in untreated control culture medium, 1182pg/ml (Figure 3.4 D, compare bar 1 to bar 2). Furthermore, treatment with OA 10 µM slightly increased 2-AG to 1245pg/ml, whereas at a concentration 50µM, 2-AG level was approximately the same, 1158pg/ml compared to nonirradiated control culture medium (Figure 3.4 D, compare bar 1 to bars 3 and 5). However, when OA10 µM or 50 µM treated culture medium were irradiated, 2-AG levels increased to 1201pg/ml and 1296pg/ml in dose dependent manner compared to irradiated control culture medium (Figure 3.4 D, compare bar 2 to bars 4 and 6).

1-AG levels in the supernatant of untreated control medium were measured to be 3025pg/ml (Figure 3.4 E, bar 1). A very small decrease in 1-AG level was observed after UV radiation compared to nonirradiated control in dose dependent manner medium (Figure 3.4 E, compare bar 1 to bar to bar 2). Treatment with OA either 10 µM or 50 µM did not show any remarkable changes in 1-AG levels (3054pg/ml and 3354pg/ml) compared to the untreated control in dose dependent manner medium (Figure 3.4 E, compare bar 1 to bars 3 and 5). UVR-stimulation of these treated in dose dependent manner medium increased 1-AG protein level in dose dependent manner to 3049pg/ml and 3257pg/ml compared to irradiated control medium (Figure 3.4 E, compare bar 2 to bars 4 and 6).
Figure 3.4 Effect of OA treatment on endocannabinoids and NAE levels in HaCaT keratinocytes culture medium in response to UV radiation. As described in Material and Methods, cells were treated either with OA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Medium was collected 24h post UVR. Quantification of endocannabinoids and NAE in HaCaT keratinocytes was carried out using LC-MS/MS analysis. Data shown as mean of n = 2 experiments. No statistical analysis was performed.
3.4.5. Quantification of endocannabinoids and NAE in cell culture medium from irradiated and non-irradiated HaCaT keratinocytes treated with DHA.

Figure 3.5 A, showed that the base line of OEA in untreated medium was estimated to be 541pg/ml (Figure 3.5 A, bar 1). DHA 10µM or 50µM treatment dose dependently increased OEA level in HaCaT cultural medium to 624pg/ml and 681pg/ml compared to untreated control medium (Figure 3.5 A, compare bar 1 to bar 3 and 5). OEA level was significantly increased in irradiated control medium cells to 767pg/ml ($P = 0.005$) compared to the nonirradiated control medium (Figure 3.5 A, compare bar 1 to bar 2). Similar effect but, no significant difference was seen when DHA 10µM (840pg/ml) or 50µM (830pg/ml) treated medium were subjected to UVR (Figure 3.5 A, compare bar 2 to bars 4 and 5) compared to irradiated control medium.

STEA levels in untreated control medium were found to be 5168pg/ml (Figure 3.5 B, bar 1). UV light significantly reduced STEA level in irradiated control medium to 2501pg/ml ($P = 0.001$) compared to nonirradiated control medium (Figure 3.5 B, compare bar 1 to bar 2). Treatment with DHA 10µM also significantly decreased STEA level to 2667pg/ml ($P = 0.001$) compared to untreated control medium (Figure 3.5 B, compare bar 1 to bar 3). However, DHA 50µM induced only small decrease (4668pg/ml) in STEA level compared to untreated control medium (Figure 3.5 B, compare bar 1 to bar 5). Both concentrations of DHA (10µM or 50µM) increased STEA protein levels to 3334pg/ml when they were irradiated compared to the irradiated control medium (Figure 3.5 B, compare bar 2 to bars 4 and 6).

DHEA levels in untreated control medium were measured to be 136pg/ml (Figure 3.5 C, bar 1), and this level was quite similar in the irradiated control medium, 130pg/ml (Figure 3.5 C, bar 2). However, no significant differences were noticed in DHEA level in non-irradiated medium treated with DHA 10 µM (130pg/ml) or 50 µM (144pg/ml) (Figure 3.5 C, compare bar 1 to bars 3 and 5) compared to untreated control medium. DHA 10µM (142pg/ml) and 50µM (144pg/ml) also have no effect on the irradiated medium compared to the irradiated control medium (Figure 3.5 C, compare bar 2 to bars 4 and 6).
2-AG levels in untreated control medium were assessed to be 1161pg/ml (Figure 3.5 D, bar 1) whereas, slight increase in 2-AG levels were observed in culture medium treated with DHA 10µM or 50µM, 1207pg/ml and 1212pg/ml respectively, compared to untreated control medium (Figure 3.5 D, compare bar 1 to bars 3 and 5). Moreover, 2-AG level was also increased after UVR stimulation up to 1336pg/ml compare to nonirradiated control medium (Figure 3.5 D, compare bar 1 to bar 2). However, 2-AG level was dropped to 1094pg/ml in UVR-stimulated medium treated with DHA 10µM and then increased again to 1363pg/ml with DHA 50µM compared to irradiated control medium (Figure 3.5 D, compare bar 2 to bars 4 and 6).

1-AG levels in the untreated control medium were considered to be 3072pg/ml (Figure 3.5 E, bar 1) whereas, in cell culture medium treated with 10µM or 50µM DHA, 1-AG levels were increased to 3153pg/ml and 3635pg/ml compared to untreated control medium (Figure 3.5 E, compare bar 1 to bars 3 and 5). After UVR stimulation, 1-AG was elevated up to 3712pg/ml but, this level was then dropped to 3414pg/ml and 3348pg/ml UVR-stimulated medium treated with DHA 10µM or 50µM respectively (Figure 3.5 E, compare bar 2 to bars 4 and 6).
Figure 3.5 Effect of DHA treatment on endocannabinoids and NAE levels in HaCaT keratinocytes culture medium in response to UV radiation. As described in Material and Methods, cells were treated either with DHA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Medium was collected 24h post UVR. Quantification of endocannabinoids and NAE in HaCaT keratinocytes was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
3.4.6. Quantification of endocannabinoids and NAE in cell culture medium from irradiated and non-irradiated HaCaT keratinocytes treated with EPA.

OEA levels in HaCaT cultural medium of untreated control cells were found to be 425pg/ml (Figure 3.6 A, bar 1). This level slightly increased in a dose dependent manner to 407pg/ml and 437pg/ml in cell culture medium treated with EPA 10µM or 50µM respectively, compared to untreated control HaCaT medium (Figure 3.6 A, compare bar 1 to bars 3 and 5). However, UVR significantly increased OEA level in irradiated control up to 555pg/ml compared to nonirradiated control medium \((P = 0.001)\) (Figure 3.6 A, compare bar 1 to bar 2). This effect was significantly enhanced when EPA 10µM (407pg/ml) \((P = 0.002)\) or 50µM (500pg/ml) \((P = 0.008)\) treated medium were irradiated compared to irradiated control medium (Figure 3.6 A, compare bar 2 to bars 4 and 6).

STEA levels in untreated control medium were calculated to be 2667pg/ml (Figure 3.6, B bar 1). Treatment with EPA10µM or 50µM increased STEA level to 3001pg/ml and 2834pg/ml respectively, compared to untreated control medium (Figure 3.6 B, compare bar 1 to bars 3 and 5). Compared to nonirradiated control medium, UV light increased STEA level in the irradiated control medium to 3167pg/ml (Figure 3.6 B, Compare bar 1 to bar 2). STEA level was then down-regulated to 2167pg/ml in irradiated medium treated with EPA 10µM, but increased again to 3667pg/ml as EPA 50µM was tested against UVR (Figure 3.6 B, compare bar 2 to bars 4 and 6).

DHEA levels in untreated control medium were estimated to be 140pg/ml (Figure 3.6 C, bar 1), this level was slightly reduced in irradiated control medium to 132pg/ml (Figure 3.6 C, bar 2). However, no significant changes were noticed in DHEA level at EPA 10 µM (137pg/ml) or 50 µM (132pg/ml) treated groups compared to untreated control medium (Figure 3.6 C, compare bar 1 to bars 3 and 5). Also, no major changes were observed in DHEA levels, 132pg/ml and 137pg/ml respectively, when EPA 10µM and 50µM treated medium were examined against UVR compared to irradiated control medium (Figure 3.6 C, compare bar 2 to bars 4 and 6).

2-AG levels in untreated control medium were assessed to be 1258pg/ml and there was no noticeable change in its level in culture medium treated with EPA 10µM or 50µM,
1171pg/ml and 1282pg/ml, respectively (Figure 3.6 D, compare bar 1 to bars 3 and 5). UVR stimulation did not induce any major change in 2-AG (1148pg/ml) compared to nonirradiated control culture medium (Figure 3.6 D, compare bar 1 to bar 2). However, 2-AG levels increased to 1201pg/ml and 1213pg/ml in a dose dependent manner in EPA 10µM or 50µM treated culture medium with UV radiation compared to the irradiated control culture medium (Figure 3.6 D, compare bar 2 to bars 4 and 6).

1-AG levels in untreated control medium were estimated to be 3058pg/ml (Figure 3.3 E, bar 1). Whereas, in HaCaT culture medium treated with EPA 10µM or 50µM 1-AG levels increased to 3498pg/ml and significantly to 3833pg/ml ($P = 0.022$) respectively, in a dose dependent manner compared to untreated control culture medium (Figure 3.6 E, compare bar 1 to bar 3 and 5). After UV radiation, 1-AG level in irradiated control cells was calculated to be 3111pg/ml, which is slightly higher than its level in nonirradiated control culture medium (Figure 3.6 E, compare bar 1 to bar 2). However, this level increased up 3688pg/ml and to 3521pg/ml respectively in UVR-stimulated culture medium treated with EPA 10µM or 50µM (Figure 3.6 E, compare bar 2 to bars 4 and 6).
Figure 3.6 Effect of EPA treatment on endocannabinoids and NAE levels in HaCaT keratinocytes culture medium in response to UV radiation. As described in Material and Methods, cells were treated either with EPA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Medium was collected 24h post UVR. Quantification of endocannabinoids and NAE in HaCaT keratinocytes was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
Table 3.1. Summary of the significant findings in HaCaT keratinocytes cells and their cell culture medium in response to DHA and/or UVR. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control using one way (ANOVA) followed by Tukey post hoc test.
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Table 3.2. Summary of the significant findings in HaCaT keratinocytes cells and their cell culture medium in response to EPA and/or UVR. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control using one way (ANOVA) followed by Tukey post hoc test.
3.4.7. Quantification of the endocannabinoids and other NAE in irradiated and non-irradiated 46BR.IN fibroblasts treated with OA.

In general, because of N= 2 in OA experiments (Figures 3.25-3.31), statistically it is not possible to assess if there are any significant differences in endocannabinoid congeners level between the different groups in those experiments.

Compared to untreated control cells STEA levels in nonirradiated control group cells were measured to be 2151 pg/mg (Figure 3.7 A, bar 1). Compared to the untreated control cells, STEA level was increased up to 5835 pg/mg in nonirradiated cells treated with 10µM OA and to 3494 pg/mg in the cells that were treated with 50µM of the same fatty acid (Figure 3.7 A compare bar 1 to bars 3 and 5). However, a 2-fold increase was seen in STEA after UVR exposure in the control cells (4757 pg/mg) (Figure 3.7 A bar 2). Whereas, treatment of the irradiated cell with OA 10µM or 50µM increased STEA level up to 4238 pg/mg and 6251 pg/mg respectively, compare to irradiated control cells (Figure 3.7 A, compare bar 2 to bars 4 and 6).

Figure 3.7 B, presented some changes in PEA levels when untreated control cells were compared with OA and UVR treated cells. PEA level in nonirradiated control group was 2958 pg/mg (Figure 3.7 B bar 1). However, this level was increased in irradiated control cells up to 5123 pg/mg, (Figure 3.7 B, bar 2). More elevation in PEA level (7522 pg/mg) was noticed after the incubation with 10µM OA alone (Figure 3.7 B, bar 3). While, this level was dropped to 2809 pg/mg (Figure 3.7 B, bar 5) when the OA concentration was increased to 50µM. These increases in PEA level were also seen when the treated samples were combined with UVR; 6527 pg/mg in bar 4 and 8596 pg/mg in bar 6 compared to the irradiated control in bar 2.

OEA levels in untreated cells were 2259 pg/mg. Incubation of the 46BR.IN fibroblasts with OA 10µM for 72h increased OEA level slightly to 3612 pg/mg, whereas, this level was decreased to 2124 pg/mg with OA 50µM (Figure 3.7 C, compare bar 1 to bars 3 and 5). Likewise, UVR elevated OEA level in irradiated control cells to 5367 pg/mg compare to nonirradiated control cells (Figure 3.7 C, compare bar 1 to bar 2). However,
this effect was reversed when OA10µM (2712 pg/mg) or 50µM (3126 pg/mg) treated cells were irradiated (Figure 3.7 C, compare bar 2 to bars 4 and 6).

DHEA levels in untreated control cells were estimated to be 323 pg/mg (Figure 3.7 D, bar 1). Whereas, DHEA level dramatically increased in irradiated control cells up to 732 pg/mg (Figure 3.7 D, bar 2). Moreover, incubation of OA 10µM or 50µM treated cells also raised DHEA levels to 695 pg/mg and 411pg/mg respectively, compared to nonirradiated control cells (Figure 3.7 D, compare bar 1 to bars 3 and 5). However, DHEA was decreased to 339 pg/mg and 625 pg/mg respectively, when OA10µM or 50µM treated cells were irradiated compared to irradiated control cells (Figure 3.7 C, compare bar 2 to bars 4 and 6).

AEA levels were found to be 323 pg/mg in untreated control cells, however, in irradiated control cells UVR increased this level up to 488 pg/mg (Figure 3.7 E, compare bar 1 to bar 2). Treatment with OA10µM increased AEA level up to 556 pg/mg while, in OA 50µM AEA level was decreased to 343 pg/mg, compared to untreated control cells (Figure 3.7 E, compare bar 1 to bars 3 and 5). AEA level was also decreased to 339 pg/mg in irradiated treated with OA10µM, in contrast, OA 50µM in irradiated cells, raised AEA up to 625 pg/mg compared to irradiated control cells (Figure 3.7 E, compare bar 2 to bars 4 and 6).

2-AG levels in untreated control cells were estimated to be 753 pg/mg (Figure 3.7 F, bar 1). UVR stimulation sharply increased 2-AG level up to 2562 pg/mg (Figure 3.7 F, bar 2). When these cells were incubated with 10µM or 50µM OA for 72h, 2-AG levels were increased to 1389 pg/mg and 1096 pg/mg respectively compared to untreated control cells (Figure 3.7 F, compare bar 1 to bars 3 and 5). However, 2-AG level was reduced in UVR-stimulated cells treated with OA 10µM to 1780 pg/mg but, when the same cells were treated with OA 50µM 2-AG level increased up to 3126 pg/mg compared to the irradiated control cells (Figure 3.7 F, compare bar 2 to bars 4 and 6).

In untreated control cells 1-AG levels were found to be 753pg/mg whereas, this level increased to 2684pg/mg in irradiated control cells (Figure 3.7 G, compare bar 1 to bar 2). Treatment with OA 10µM or 50µM in addition, increased 1-AG levels to 1667pg/mg
and 1096pg/mg compared to untreated control cells (Figure 3.7 G, compare bar 1 to bars 3 and 5). However, 1-AG level was reduced to 1865pg/mg in UVR-stimulated cells treated with OA 10µM compared to irradiated control cells but, when the OA 50µM was used 1-AG level increased up to 3126 pg/mg in these cells compared to their control cells (Figure 3.7 G, compare bar 2 to bars 4 and 6).
Figure 3.7 Effect of OA treatment on endocannabinoids and NAE levels in 46BR.IN fibroblasts in response to UV radiation. As described in Materials and Methods, cells were treated either with OA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Cells were harvested with 0.25% trypsin-EDTA solution 24h post UVR. Quantification of endocannabinoids and NAE in 46BR.IN fibroblasts was carried out using LC-MS/MS analysis. Data shown as mean of n = 2 experiments. No statistical analysis was performed.
3.4.8. Quantification of the endocannabinoids and other NAE in irradiated and non-irradiated 46BR.IN fibroblasts treated with DHA

STEA levels were calculated to be 3741pg/mg in untreated control cells (Figure 3.8 A, bar 1). In addition, when these cells were incubated with DHA 10µM or 50µM for 72h, STEA levels were increased to 5459pg/mg and 5984pg/mg, respectively (Figure 3.8 A, compare bar 1 to 3 and 5). STEA level in the irradiated control was slightly lower (3287pg/mg) compared to its level in nonirradiated control cells (Figure 3.8 A, compare bar 1 to bar 2). In UVR-stimulated cells treated with DHA 10µM or 50µM STEA levels were significantly increased in dose dependent manner to 7881pg/mg ($P = 0.001$) or 7153pg/mg ($P = 0.002$), respectively compared to the irradiated control (Figure 3.8 A, compare bar 2 to bars 4 and 6).

Figure 3.48 showed that there were no significant differences in PEA levels between nonirradiated (2276pg/mg) and irradiated (2343pg/mg) control cells (Figure 3.8 B, compare bar 1 to bar 2). PEA level was slightly increased up to 3237pg/mg after the incubation with 10µM DHA alone (Figure 3.8 B, bar 3), while, this level dropped to 2844pg/mg (Figure 3.8 B, bar 5) when DHA concentration was increased to 50µM. The same trend was also seen when the treated cells were exposed to UVR. PEA level was significantly increased up to 4658pg/mg ($P = 0.001$) and 3646pg/mg ($P = 0.03$) with DHA10µM and 50µM respectively, compared to the irradiated control (Figure 3.8 B, compare bar 2 to bar 4 and 6).

Figure 3.8 C showed that OEA levels in untreated control cells were assessed to be 1791pg/mg (Figure 3.8 C, bar 1). However, this level was decreased to 1407pg/mg in irradiated control cells (Figure 3.8 C, bar 2). Treatment with DHA10µM or 50µM significantly increased OEA levels up to 3394pg/mg ($P = 0.001$) and 2942pg/mg ($P = 0.01$) respectively, compared to untreated control cells (Figure 3.8 C, compare bar 1 to bars 3 and 5). This effect was in addition, enhanced when DHA10µM or 50µM treated cells were irradiated, OEA levels were significantly further increased up to 4587pg/mg ($P = 0.001$) and 4107pg/mg ($P = 0.001$) respectively, compared to irradiated control cells (Figure 3.8 C, compare bar 2 to bars 4 and 6).
DHEA levels in untreated control cells were estimated to be 345 pg/mg (Figure 3.8 D, bar 1). Whereas, in irradiated control cells this level was dropped to 309 pg/mg (Figure 3.8 D, bar 2). Treatment with DHA 10 µM or 50 µM significantly up-regulated DHEA protein levels in dose dependent manner up to 631pg/mg ($P = 0.016$) and 1085pg/mg ($P = 0.001$) compared to the untreated control cells (Figure 3.8 D compare bar 1 to bars 3 and 5). Likewise, similar trend was observed when the treated groups combined with UVR, OEA levels were significantly increased to 1165 pg/mg ($P = 0.001$) and 1184 pg/mg ($P = 0.001$) in DHA 10µM or 50µM, respectively compared to irradiated control cells (Figure 3.8 D, compare bar 2 to bars 4 and 6).

Figure 3.8 E showed AEA levels at 517 pg/mg in untreated control cells, this level was slightly decreased after UVR to 494 pg/mg in irradiated control cells (Figure 3.8 E, compare bar 1 and bar 2). Treatment with DHA10µM or 50µM increased AEA level up to 663 pg/mg and 632 pg/mg, respectively compared to untreated control cells (Figure 3.8 E, compare bar 1 to bars 3 and 5). Furthermore, AEA levels were significantly further increased up to 981pg/mg ($P = 0.001$) and 841pg/mg ($P = 0.001$) respectively in irradiated cells treated with DHA 10µM or 50µM compared to irradiated control cells (Figure 3.8 E, compare bar 2 to bars 4 and 6).

2-AG levels in the untreated control cells were found to be 1680 pg/mg (Figure 3.8 F, bar 1). Whereas, UVR stimulation significantly increased this level up to 3544pg/mg ($P = 0.001$) in the irradiated control cells (Figure 3.8 F, bar 2). Also, when these cells were treated with 10µM or 50µM DHA for 72h, 2-AG levels were increased to 2290 pg/mg and 2037pg/mg, respectively compared to the untreated control cells (Figure 3.8 F, compare bar 1 to bars 3 and 5). However, in comparison to the irradiated control cells, 2-AG level was dramatically increased up to 5009 pg/mg ($P = 0.002$) in UV-stimulated cells treated with DHA 10µM (Figure 3.8 F, compare bar 2 to bar 4). However, 2-AG was decreased to 3223 pg/mg, when DHA 50µM was used in combination with UVR compared to the irradiated control cells (Figure 3.8 F, compare bar 2 to bar 6).

1-AG levels in the untreated control cells were measured to be 1236 pg/mg, whereas, UVR significantly increased 1-AG level to 2985 pg/mg ($P = 0.001$) (Figure 3.8 G,
compare bar 1 to bar 2). When these cells were treated with 10µM or 50µM DHA for 72h, 1-AG level increased to 1700 pg/mg and 1433 pg/mg respectively compared to the untreated control cells (Figure 3.8 G, compare bar 1 to bars 3 and 5). Moreover, 1-AG levels were additional increased to 3573 pg/mg and 3517pg/mg in UVR-stimulated cells treated with DHA 10µM or 50µM respectively compared to the irradiated control cells (Figure 3.8 G, compare bar 2 to bars 4 and 6).
Figure 3.8 Effect of DHA treatment on endocannabinoids and NAE levels in 46BR.IN fibroblasts in response to UV radiation. As described in Material and Methods, cells were treated either with DHA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Cells were harvested with 0.25% trypsin-EDTA solution 24h post UVR. Quantification of endocannabinoids and NAE in 46BR.IN fibroblasts was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
3.4.9. Quantification of the endocannabinoids and other NAE in irradiated and non-irradiated 46BR.IN fibroblasts treated with EPA.

STEAs levels in the control cells were calculated to be 5427 pg/mg, whereas, this level was slightly higher, 5868 pg/mg in the irradiated control cells (Figure 3.9 A, compare bar 1 to bar 2). Incubation of these cells with EPA 10µM or 50µM for 72h reduced STEA levels to 4063 pg/mg and 4864 pg/mg, respectively compared to the untreated control cells (Figure 3.9 A, compare bar 1 to 3 and 5). However, treatment with EPA 10µM or 50µM did not induce any remarkable changes in STEA levels in UV irradiated cells, compared to the irradiated control cells (Figure 3.9 A, compare bar 2 to bars 4 and 6).

Figure 3.9 B, illustrated that PEA levels in the control cells were measured to be 3336 pg/mg, while, this level was slightly decreased to 3174 pg/mg in the irradiated control cells (Figure 3.9 B, compare bar 1 to bar 2). Two different concentrations of EPA (10µM or 50µM) were used. PEA levels decreased to 2340 pg/mg and 2378 pg/mg, respectively in dose dependent manner compared to the untreated control cells (Figure 3.9 B, compare bar 1 to bars 3 and 5). Whereas, in the irradiated cells treated with EPA 10µM or 50µM, PEA levels increased to 3402 pg/mg and 3860 pg/mg respectively, in a dose dependent manner compared to the irradiated control cells (Figure 3.9 B, compare bar 2 to bars 4 and 6).

OEA levels in the untreated cells were estimated to be 3484 pg/mg, while UV radiation down-regulated this level to 2591 pg/mg (Figure 3.9 C, compare bar 1 to bar 2). Incubation of 46BR.IN fibroblastic cells with EPA 10µM or 50µM for 72h decreased the OEA level significantly to 1886 pg/mg (P = 0.015) and marginally to 2782 pg/mg respectively compared to the untreated control cells (Figure 3.9 C, compare bar 1 to bars 3 and 5). However, OEA level was increased to 3070 pg/mg in irradiated cells treated with EPA 10µM (Figure 3.9 C, bar 4), but with EPA 50µM this level was dropped to 1940 pg/mg compared to the irradiated control cells (Figure 3.9 C, compare bar 2 to bars 4 and 6).

DHEA levels in the untreated control cells were considered to be 255 pg/mg, whereas, this level was raised up to 327 pg/mg in the irradiated control cells (Figure 3.9 D,
compare bar 1 to bar 2). Treatment with EPA 10µM decreased DHEA level to 214 pg/mg whilst, EPA 50µM significantly increased DHEA level to 452pg/mg ($P = 0.008$) compare to the untreated control cells (Figure 3.9 D, compare bar 1 to bars 3 and 5). This figure also illustrated that DHEA significantly increased to 656 pg/mg ($P = 0.001$) and 604 pg/mg ($P = 0.001$), respectively after EPA 10µM or 50µM treated cells were irradiated compared to the irradiated control cells (Figure 3.9 D, compare bar 2 to bars 4 and 6).

Figure 3.9 E showed AEA level at 32pg/mg in the untreated control cells (Figure 3.9 E, bar 1). UVR stimulation, did not induce any major change in AEA level in the irradiated control cells (30 pg/mg) (Figure 3.9 E, bar 2). Moreover, treatment of the nonirradiated cells with EPA, either 10µM or 50µM also did not produce any noticeable change in AEA level, 30 pg/mg and32 pg/mg, respectively compared to the untreated control cells (Figure 3.9 E, compare bar 1 to bars 3 and 5). In addition, when the UV irradiated cells were treated with the same fatty acids either 10µM or 50µM no significant changes were seen in AEA level in this experiment (32pg/mg and 33pg/mg) (Figure 3.9 E compare bar 2 and bars 4 and 6).

2-AG levels in nonirradiated control cells were estimated to be 3144 pg/mg however, in the irradiated control cells this level significantly increased about 3-fold to 10501 pg/mg ($P = 0.001$) (Figure 3.9 F, compare bar 1 to bar 2). No significant differences were seen when these cells were incubated with EPA 10µM or 50µM for 72h, 3518 pg/mg and 3453 pg/mg respectively (Figure 3.9 F, compare bar 1 to bars 3 and 5). In addition, when these cells were treated with EPA 10µM, 2-AG level was approximately similar to its level in the irradiated control cells but, when EPA 50µM was used 2-AG level slightly decreased to 9025 pg/mg (Figure 3.9 F, compare bar 2 to bars 4 and 6).

1-AG levels in the untreated control cells were assessed to be 3059 pg/mg (Figure 3.9 G, bar 1). Treatment with EPA 10µM or 50µM for 72h decreased 1-AG level to 2835 pg/mg and 3853 pg/mg respectively (Figure 3.9 G, compare bar 1 to bars 3 and 5). UVR stimulation significantly increased 1-AG level to 10377 pg/mg ($P = 0.001$) compared to nonirradiated control cells (Figure 3.9 G, compare bar 1 to bar 2). However, 1-AG level was slightly increased to 11251 pg/mg in UVR-stimulated cells treated with EPA 10µM.
(Figure 3.9 G, compare bar 2 to bar 4) and then reduced to 7818 pg/mg when these cells treated with EPA 50µM (Figure 3.9 G, compare bar 2 to bar 6).
Figure 3.9 Effect of EPA treatment on endocannabinoids and NAE levels in 46BR.IN fibroblasts in response to UV radiation. As described in Material and Methods, cells were treated either with EPA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Cells were harvested with 0.25% trypsin-EDTA solution 24h post UVR. Quantification of endocannabinoids and NAE in 46BR.IN fibroblasts was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
3.4.10. Quantification of the endocannabinoids and other NAE in culture medium from irradiated and non-irradiated 46BR.IN fibroblasts treated with OA

Because of N=2 in the OA experiments, statistically it is not possible to assess if there are any significant differences in endocannabinoids congeners level between the different groups in those experiments.

STEA levels in control medium were 3001 pg/ml (Figure 3.10 A, bar 1). Adding OA 10µM or 50µM to cell culture medium decreased STEA level to 2000 pg/ml and 2501 pg/ml respectively compared to the untreated control (Figure 3.10 A, compare bar 1 to bars 3 and 5). Furthermore, STEA level was decreased to 2501 in the irradiated control culture medium compared to the nonirradiated control medium (Figure 3.10 A, compare bar 1 to bar 2). Treatment with OA either 10µM increased STEA level in cell culture medium of the irradiated cells, whereas, 50µM did not induce any significant change compare to their control (Figure 3.10 A, compare bar 2 to bars 4 and 6).

Figure 3.10 B, presented DHEA level at 473 pg/ml in the untreated control medium (Figure 3.10 B, bar 1). This level was dropped to 359 pg/ml after UVR stimulation compared to nonirradiated culture medium (Figure 3.10 B, compare bar 1 to bar 2). Using two different concentrations of OA, either 10µM or 50µM also decreased DHEA level to 2257 pg/ml and 402 pg/ml respectively in the nonirradiated culture medium compared to their control (Figure 3.10 B, compare bar 1 to bars 3 and 5). In contrast, DHEA levels were dose dependently increased in the culture medium of irradiated cells after treatment with OA 10µM or 50µM compared to the irradiated control culture medium (Figure 3.10 B, compare bar 2 to bars 4 and 6).

2-AG levels in the untreated control medium were calculated to be 2749 pg/ml (Figure 3.10 C, bar 1) In addition, minor downregulation in 2-AG level was noticed when the culture medium of the nonirradiated cells was incubated with OA 10µM (1952 pg/ml) or 50µM (2495 pg/ml) compared to their control (Figure 3.63 compare bar 1 to bars 3 and 5). No significant changes were seen in 2-AG level in the culture medium of irradiated cells compared to nonirradiated cell culture medium (Figure 3.10 C, compare bar 1 to bar 2). However, treatment with OA 10µM or 50µM did not induce any major changes
in 2-AG levels, 2580 pg/ml and 2527 pg/ml respectively compare to the medium of the irradiated control cells (Figure 3.10 C, compare bar 2 to bars 4 and 6).

1-AG levels in the untreated control culture medium were 34507 pg/ml whereas, this level was decreased to 25505 pg/ml in the cell culture medium of the irradiated cells (Figure 3.10 D, compare bar 1 to bar 2). Adding OA 10µM to the cell culture medium decreased 1-AG levels to 28506 pg/ml while in those treated with 50µM 1-AG level was increased to 35007 pg/ml (Figure 3.10 D, compare bar 1 to bar 3 and bar 5). Furthermore, using the UVR in combination either with 10µM or 50µM OA increased 1-AG level in dose dependent manner to 33007 pg/ml and 400008 pg/ml respectively compared to the irradiated control culture medium (Figure 3. 10 D, compare bar 2 to bar 4 and bar 6).
Figure 3.10 Effect of OA treatment on endocannabinoids and NAE levels in 46BR.IN fibroblasts culture medium in response to UV radiation. As described in Materials and Methods, cells were treated either with OA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Medium was collected 24h post UVR. Quantification of endocannabinoids and NAE in 46BR.IN fibroblasts was carried out using LC-MS/MS analysis. Data shown as mean of n = 2 experiments. No statistical analysis was performed.
3.4.11. Quantification of the endocannabinoids and other NAE in culture medium from irradiated and non-irradiated 46BR.IN fibroblasts treated with DHA.

Figure 3.11 A, showed STEA level at 5001 pg/ml in cell cultural medium of untreated control cells. However, after UVR stimulation this level was significantly reduced to 2334 pg/ml in cell culture of the irradiated control cells compared to the nonirradiated medium \((P = 0.001)\) (Figure 3.11 A, compare bar 1 to bar 2). Treatment with DHA, either 10µM or 50µM also significantly decreased STEA level in dose dependent manner to 2834 pg/ml \((P = 0.001)\) and 2334 pg/ml \((P = 0.001)\) compared to untreated culture medium (Figure 3.11 A, compare bar 1 to bars 3 and 5). In contrast, the combination between the DHA 10µM or 50µM and UVR increased STEA level to 3001 pg/ml and significantly to 4001 pg/ml \((P = 0.008)\), respectively compared to cell culture medium of irradiated control cells (Figure 3.65 compare bar 2 to bars 4 and 6).

DHEA levels were estimated to be 352 pg/ml in the untreated control culture medium. This level was however, increased to 504 pg/ml after UVR stimulation in the irradiated control culture medium (Figure 3.11 B, Compare bar 1 to bar 2). DHA 10µM slightly reduced DHEA level to 320 pg/ml while, DHA 50µM, increased it to 462 pg/ml compared to the culture medium of nonirradiated cells (Figure 3.11 B, compare bar 1 to bars 3 and 5). Similar effect was observed when DHA 10µM (431 pg/ml) or 50µM (611 pg/ml) treated culture medium were irradiated, compared to the irradiated control culture medium (Figure 3.11 B, compare bar 2 to bars 4 and 6).

In untreated control medium, 2-AG levels were assessed to be 2283 pg/ml whereas, when this culture medium was treated with 10µM or 50µM DHA, marginally increases were seen in 2-AG levels, 2699 pg/ml and 2486 pg/ml, respectively compared to the untreated control culture medium (Figure 3.11 C, compare bar 1 to bars 3 and 5). In cell culture of the irradiated control cells 2-AG level was slightly increased to 2766 pg/ml compared to the nonirradiated medium (Figure 3.11 C, compare bar 1 to bar 2). In addition, when the treated medium were irradiated, 2-AG levels increased to 3215 pg/ml and 3965 pg/ml with DHA 10µM and 50µM, respectively compared to the cell cultural of the irradiated control cells (Figure 3.11 C, compare bar 2 to bars 4 and 6). Figure 3.11
D, showed 1-AG levels at 40341 pg/ml in the untreated medium (Figure 3.11 D, bar 1). UVR stimulation increased this level to 59012 pg/ml compared to the nonirradiated medium (Figure 3.11 D, compare bar 1 to bar 2). Treatment with DHA10µM and 50µM decreased this level to 36341 pg/ml and 38508 pg/ml, respectively compared to the untreated medium (Figure 3.11 D, compare bar 1 to bars 3 and 5). DHA 10µM or 50µM 1-AG also decreased 1-AG level to 37674 pg/ml and 44342 pg/ml, respectively when the DHA treated medium was irradiated, compared to the irradiated control medium (Figure 3.11 D, compare bar 2 to bars 4 and 6).
Figure 3.11 Effect of DHA treatment on endocannabinoids and NAE levels in 46BR.IN fibroblasts culture medium in response to UV radiation. As described in Material and Methods, cells were treated either with DHA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Medium was collected 24h post UVR. Quantification of endocannabinoids and NAE in 46BR.IN fibroblasts was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
3.4.12. Quantification of the endocannabinoids and other NAE in culture medium from irradiated and non-irradiated 46BR.IN fibroblasts treated with EPA.

STEAs levels were found to be 3001 pg/ml in cell cultural medium of the untreated control cells (Figure 3.12 A, bar 1). However, this level was reduced after UVR stimulation to 2167 pg/ml in the irradiated control medium (Figure 3.12 A, compare bar 1 to bar 2). Treatment with EPA, either 10µM or 50µM decreased STEA level to 2167 pg/ml (Figure and 2667 pg/ml, respectively. In contrast, the combination between the EPA and UVR increased the STEA level in dose dependent way to 2334 pg/ml and 3001 pg/ml, respectively compare to the irradiated control medium (Figure 3.12 A, compare bar 2 to bars 4 and 6).

Figure 3.12 B, showed DHEA levels at 223 pg/ml in the cell culture of untreated control cells. After UVR stimulation, this level was almost the same, 247 pg/ml in the irradiated control medium (Figure 3.12 B, compare bar 1 to bar 2). In compare to the untreated culture medium, treatment with EPA 10µM or 50µM did not produce any remarkable change in DHEA levels, 247 pg/ml and 226 pg/ml respectively (Figure 3.12 B, compare bar 1 to bars 3 and 5). In addition, the combination between EPA and UVR also did not show any significant changes, as DHEA levels were 258 pg/ml and 303 pg/ml after EPA 10µM and 50µM treatment, respectively (Figure 3.12 B, compare bar 2 to bars 4 and 6).

2-AG levels were estimated to be 2298 pg/mg in cell culture medium of untreated control cells (Figure 3.12 C, bar 1). However, UVR stimulation significantly increased this level to 4004 pg/ml in the irradiated control culture medium \((P = 0.026)\) (Figure 3.12 C, compare bar 1 to bar 2). EPA10µM or 50µM in addition, increased 2-AG protein level to 3309 pg/ml and significantly to 4311 pg/ml \((P = 0.009)\), respectively compared to the untreated culture medium (Figure 3.12 C, compare bar 1 to bars 3 and 5). Compared to the irradiated control culture medium, 2-AG level decreased to 3319 pg/mg but increased again to 4499 pg/ml in cell culture medium of irradiated cells treated with EPA 10µM and 50µM, respectively (Figure 3.12 C, compare bar 2 to bars 4 and 6).
In untreated control medium, 1-AG levels were measured to be 47343 pg/ml, but in irradiated control medium this level was slightly decreased to 36841 pg/ml (Figure 3.12 D, compare bar 1 to bar 2). Adding EPA 10μM and 50μM into cell culture medium of the nonirradiated cells, reduced 1-AG levels to 31173 pg/ml and 37174 pg/ml, respectively. In contrast, UVR in combination with 10μM or 50μM EPA increased 1-AG levels to 46676 pg/ml and 41008 pg/ml, respectively, compared to the irradiated control medium (Figure 3.12 D, bar 2 to bars 4 and 6).
Figure 3.12 Effect of EPA treatment on endocannabinoids and NAE levels in 46BR.IN fibroblasts culture medium in response to UV radiation. As described in Materials and Methods, cells were treated either with EPA10 or 50 µM for 72h and then they were irradiated with 15J/cm² UVB. Medium was collected 24h post UVR. Quantification of endocannabinoids and NAE in 46BR.IN fibroblasts was carried out using LC-MS/MS analysis. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
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**Table 3.3.** Summary of the significant findings in 46 BR.IN fibroblasts cells and their cell culture medium in response to DHA and/or UVR. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control using one way (ANOVA) followed by Tukey post hoc test.
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**Table 3.4.** Summary of the significant findings in 46 BR.IN fibroblasts cells and their cell culture medium in response to EPA and/or UVR. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control using one way (ANOVA) followed by Tukey post hoc test.
3.5. Discussion

3.5.1. Determination of the OA and UVR effects on the intracellular and extracellular levels of endocannabinoids and NAE in HaCaT Keratinocytes and 46BR.IN fibroblasts.

As non-essential monounsaturated fatty acid rich in cell membrane, lacking the ability to be metabolized to DHA or EPA (Grammatikos et al., 1994), OA was selected to be a control fatty acid in the present study. Our results showed that the UVR induced up-regulation in STEA, PEA, OEA and DHEA was inhibited when the irradiated HaCaT keratinocytes were treated with OA. In 46BR.IN fibroblasts the most noticeable effect was that the intracellular levels of STEA, PEA, OEA, DHEA, AEA, 2-AG and 1-AG were increased after UVR stimulation. However, this effect was inhibited and enhanced with OA 10µM and 50µM respectively.

Only a few mediators were detected in the culture medium from HaCaT keratinocytes and 46BR.IN fibroblasts, including STEA, (OEA HaCaT keratinocyte culture medium only), DHEA and 2-AG. Fluctuated effects ranging from minor decrease or increase were observed on the extracellular levels of these mediators after OA treatment and or UVR exposure. In general, the fluctuation effects of OA treatment on the levels of the above mentioned mediators could be attributed to the inability of OA to form these mediators in HaCaT keratinocytes or 46BR.IN fibroblasts. Apart from this, the above mentioned results related to OA treatment were obtained from experiments carried out only twice; therefore concrete conclusions cannot be drawn from these observations.

3.5.2. Determination of the n-3 PUFA effects on the intracellular and extracellular levels of the endocannabinoids and NAE in HaCaT Keratinocytes and 46BR.IN fibroblasts in response to ultraviolet radiation.

Data from this study showed that DHA treatment increased intracellular levels of PEA, DHEA and AEA in HaCaT keratinocytes, while mediators such OEA, LEA and 1-AG were found to be decreased after DHA treatment in these cells. However, DHA has no major effects on intracellular levels of STEA and 2-AG in HaCaT keratinocytes.
Furthermore, DHA increased the extracellular levels of OEA, 2-AG and 1-AG in HaCaT keratinocytes and has no major effects on STEA and DHEA extracellular levels. However, EPA treatment was found to reduce the intracellular levels of PEA, LEA, DHEA, 2-AG and 1-AG in HaCaT keratinocytes. EPA treatment increased only OEA level in HaCaT cells and has no remarkable effects on intracellular levels of STEA and AEA.

In 46BR.IN fibroblasts, the intracellular levels of STEA, PEA, OEA, LEA, DHEA, AEA, 2-AG and 1-AG was found to be increased after DHA treatment. DHA also increased the extracellular levels of OEA, 2-AG and 1-AG in these cells, whereas, the extracellular levels of mediators such STEA and DHEA did not change by DHA. In contrast to DHA, EPA treatment decreased STEA, PEA, OEA, DHEA and 1-AG intracellular levels in 46BR.IN fibroblasts; EPA had no effects on AEA and 2-AG intracellular levels in 46BR.IN fibroblasts. Similar to DHA, EPA increased the extracellular levels of OEA, 2-AG and 1-AG in the media of 46BR.IN fibroblasts and had no major effects on STEA and DHEA extracellular levels.

UV radiation decreased the intracellular levels of STEA, PEA and LEA in HaCaT keratinocyte and 46BR.IN fibroblasts both in the presence and absence of DHA or EPA treatments. Fluctuated effects on DHEA, AEA, 2-AG and 1-AG were observed post UV radiation but in general the intracellular levels of these mediators tended to increase after UVR exposure in HaCaT keratinocyte and 46BR.IN fibroblasts. Moreover, UVR induced up-regulation in the extracellular levels of OEA, 2-AG and 1-AG in cell cultural medium of HaCaT keratinocyte and 46BR.IN fibroblasts. However, the extracellular levels of STEA and DHEA did not change post UV radiation in the media of these cells.

Overall, the trend shown in these results is consistent with another study carried out on rodents which showed that a fish oil diet containing high levels of EPA and DHA lead to a decrease in the intracellular formation of the 2-AG, PEA, STEA and OEA but not AEA and DHEA (Artmann et al., 2008) in the liver of the test animals. Furthermore, AEA and 2-AG also have been reported to decrease in 3T3-F442A adipocytes after 72h incubation with 100µM DHA (Matias et al., 2008). One possible interpretation of these
results is that DHA can reduce cell and tissue levels of AA and AA-derived downstream metabolites, including AEA and/or AG, potentially due to substrate competition among biosynthetic enzymes (Artmann et al., 2008, Connor et al., 1990, Rapoport, 2008, Watanabe et al., 2003, Lim and Suzuki, 2001, Matias et al., 2008, Batetta et al., 2009). This competition among lipid biosynthetic enzymes utilizing fatty acids as substrates could represent an important influence on the formation of downstream lipid metabolites, including their derived endocannabinoids metabolite components. Furthermore, the acylation of endocannabinoid precursors such as membrane phospholipids NAPE and DAG by DHA could be a potential explanation for the changes observed toward DHA-derived (DHEA) in HaCaT keratinocytes and 46BR.IN fibroblasts. Alternatively, the results of the current study showed that AEA, DHEA and PEA intracellular levels increased as DHA concentration increased in the irradiated and nonirradiated HaCaT keratinocytes. Moreover, AEA and DHEA intracellular levels were significantly increased in a dose dependent manner after the irradiated and non-irradiated 46BR.IN fibroblasts were treated with DHA. These results are in agreement with another study in which it has been demonstrated that brain levels of AEA and DHEA in piglets increased with DHA treatment (Berger et al., 2001). In addition, other studies in different species have confirmed an increased formation of AEA, 2-AG and DHEA in the jejunum of the rat after fish oil diet providing high level of EPA and DHA (Artmann et al., 2008). DHA has been found in the brain, gut, retina and liver in concentrations similar or higher than those of AEA (Berger et al., 2001, Wood et al., 2010), therefore, increase its derivative DHEA level is a normal consequence. Hence, many studies demonstrated that the DHEA can be synthesized from its precursor in different types of tissues (Balvers et al., 2010, Kim et al., 2011). Moreover, another study suggested the N-docosahxaenoyl-PE as a possible precursor of DHEA (Bisogno et al., 1999). The effect of the DHA and EPA treatment on STEA, OEA, LEA and 2-AG was similar in both HaCaT keratinocytes and 46BR.IN fibroblasts after UV radiation. In general the changes recorded in their intracellular levels were minor. UV radiation in this study caused the DHEA intracellular levels to be significantly increased in DHA treated cells of both used models (AEA level was increased in 46BR.IN fibroblasts only). DHEA intracellular level
was also significantly increased after UV radiation in 46BR.IN fibroblasts treated with EPA. Moreover, the intracellular levels of 2-AG and its isomer, 1-AG were found to be significantly increased after UVR exposure either in EPA treated or untreated 46BR.IN fibroblasts.

All these findings showed that the UV radiation might have an effect on the formation of endocannabinoids and NAE. These effects can be attributed to phospholipase A2 (PLA2) activity that induced after UVR exposure (Chen et al., 1996, Cohen and DeLeo, 1993, Hanson and DeLeo, 1990), as PLA2 has been reported to be one of the possible biosynthetic routes of NAEs via N-acyl-lysoPE (Sun et al., 2004). Moreover, PLA2 has been reported to induce an increase in NAEs levels including AEA (Berdyshev et al., 2000). Moreover, UVB can phosphorylate ERK (Chang et al., 2011) and p38 mitogen-activated kinases (Bachelor et al., 2005, Kim et al., 2005) which then triggers NF-kB (Chun and Surh, 2004). Therefore, UVR could impact FAAH and NAPE-PLD proteins levels through their transcriptional aspects and gene expression. NF-kB is a translation factor that perform a key part in cellular reaction to ecological pressure such as UVR by causing or controlling the expression of particular gene (Herrlich et al., 2008). UVR-induced NF-kB initial has been revealed in several research. For example, NF-kB initial in epidermis fibroblasts has been confirmed after UVA radiation (Vile et al., 1995, Reelfs et al., 2004). Also, UVB was discovered to generate NF-kB initial in human epidermis keratinocytes (Adhami et al., 2003) and NF-kB could control FAAH gene expression (Maccarrone et al., 2003a). Furthermore, Liu et al., has recommended NF-kB as the primary translation factor manages LPS-induced AEA features (Liu et al., 2003). Moreover, NF-kB was discovered to communicate with specific proteins 1 (SP 1); the translation factor of NAPE-PLD gene (Sancéau et al., 1995). For example, p65 a subunit of NF-kB has been found to interact with SP1 (Perkins et al., 1994, Pazin et al., 1996). Therefore, UVR could regulates the expression of endocannabinoids and their congeners throughout the transcriptional factors of their genes. All these findings support our findings. These data could be the first step for future studies to define potentially beneficial relationships between the effects of n-3 PUFA on endocannabinoids and their related lipids. Also endocannabinoids modification due to
UVB exposure could be one of the defense mechanism performance by human skin cells against UVB. Therefore, the biochemical role of n-3 PUFA in respect to the interaction with the biosynthesis of endocannabinoids and NAE should be further investigated.

Apart of UVB, UVA (320-400 nm) is a possible environmental stress has obtained less attention, even though extreme sun visibility is also associated with extreme contact with UVA (320-400 nm). The UVA transfer (80 nm) is twice that of UVB (40 nm), and 90% to 95% of solar UV radiation energy that gets to the surface of our planet is UVA; only 5% to 10% of it is UVB. UVA has a longer period wavelengths than UVB and thus spreads throughout further into skin. Because its short wavelength, UVB can penetrate only the full epidermis and only 10 to 15% of it can reach the upper layer of the dermis; papilla. However, 50% of UVA are absorbed by the epidermis, while the rest penetrate the dermis up to 2mm depth (Césarini, 1996). The thickness of the epidermis is increasing with direct consequence on the UVB absorption, making the basal layer of the epidermis out of reaction with UVB radiations (Césarini, 1996). The UVA sunburn is more violaceous with prominent vasodilation, therefore, UVA-induced erythema is not representation of pigmentation or melanogenesis. In the UVB, the induced division of the keratinocytes increases the thickness of the epidermis and so the total content in melanin, whereas, in the UVA, the multiplication of keratinocytes is provided directly by stimulation of melanogenesis (McKinlay and Difffey, 1987). UVA has been proven to cause mutations in mammalian cells. For example, research in murine cell lines have confirmed that UVA can generate mutations (Hitchins et al., 1986, Lundgren and Wulf, 1988). Moreover, UVA at 365nm could be mutagenic to cultured human fibroblasts (Enninga et al., 1986). The prospective dangerous impact of UVA has also been confirmed in cultured human melanocytes. UVA (320-400 nm) was found to induce DNA single-strand breaks in cultured human melanocytes (Wenczl et al., 1998, Marrot and Meunier, 2008). UVB are essentially inducing DNA dimers which are most of the time, error free repaired. However, UVA induce DNA strand breaks much less frequently than UVB, but the strand breaks are error prone lesions (Césarini, 1996). As a consequence.

After long tern exposure the genetic code is altered equally by UVA and UVB (Césarini,
1996). UVR also has an effect on lipids in human skin where they subjected to peroxidation through indirect mechanisms after UV radiation (Césarini, 1996). UVB and UVA leading to activated molecules responsible for the production of several cytokines, mitosis inducer, vascular changes and systemic distance effects (Césarini, 1996).
CHAPTER 4: The effect of n-3PUFA on endocannabinoid metabolizing enzymes in HaCaT keratinocytes and 46BR.IN fibroblasts in response to ultraviolet radiation.

4.1. Introduction

4.1.1. Cyclooxygenase-2

COX-2 is a stress inducible enzyme, expressed in different human skin cells such as fibroblasts, macrophages and keratinocytes (Gilroy et al., 2001). In response to different stimuli such as UVB, phorbol 12-myristate 13-acetate (PMA) and interleukin-1β (IL-1β), the expression of COX-2 in these cells is stimulated. This in turn plays an important role in inflammatory and tissue injury (Gilroy et al., 2001). Moreover, UVB has been demonstrated to activate the cytosolic phospholipase A2 (cPLA2), which mediates prostaglandin synthesis in human keratinocytes during the first 6h of UVB exposure (Gresham et al., 1996). COX-2 mRNA and COX-2 protein levels have been detected in human keratinocytes following 24 h of UVB irradiation irrespectively of the presence of serum (Buckman et al., 1998). Also, COX-2 expression is significantly increased in human dermis and epidermis 24 h after UVB exposure (Rhodes et al., 2009). Moreover, a recent study demonstrated the up-regulation of COX-2 expression and protein levels in cultured HaCaT cells and in mice subjected to 30mJ/cm² UVB (Lee et al., 2013b).

COX-2 is upregulated in response to induction by multiple mitogenic and pro-inflammatory stimuli, including growth factors, cytokines, hormones, serum, hypoxia, bacterial endotoxin, and carcinogens (Smith et al., 2000, Trifan and Hla, 2003, Buckman et al., 1998). Furthermore, COX-2 is involved in pathophysiological conditions such as inflammation, pain, fever wound repair, angiogenesis, vasodilation and increased vascular permeability (Smith et al., 2000, Gilroy et al., 2001, Funk, 2001).

While AA is the normal substrate for COX-1 and COX-2 isoforms, other fatty acids can be oxygenated by both of them (Laneuville et al., 1995, Wada et al., 2007, Yuan et al., 2009). COX-2 for example, has up to 30% efficiency to oxygenate the fatty acid eicosapentaenoic acid (EPA) (20:5 n-3) compared with AA. Whereas, COX-1 efficiency to oxygenate the same fatty acid is less than 5% (Wada et al., 2007, Malkowski et al.,
COX-2 can oxygenate a more extensive array of substrates compared with COX-1, presumably because of an approximately 20% larger active site (Kurumbail et al., 1996). Also, it has been established that COX-2, but not COX-1, oxygenates the endocannabinoids 2-AG and AEA to produce respectively prostaglandin glycerol esters (PG-Gs) and prostaglandin ethanolamines (PG-EAs) (Kozak et al., 2000, Kozak et al., 2001b). 2-AG and AEA are widely distributed in mammalian tissues including human skin (Bisogno, 2008). COX-2 oxygenates endocannabinoid substrates using the same mechanism employed for AA, generating prostaglandin, thromboxane, and prostacyclin ethanoloamides, glycerol esters, and glycine (Kozak et al., 2000, Kozak et al., 2002a, Kozak et al., 2001a). The ability of COX-2 to metabolize endocannabinoid substrates suggests that this isoform may be involved in the endogenous endocannabinoid signalling system. Therefore, our study aimed to investigate the effects of UVR and n-3 PUFAs on COX-2 in relation to endocannabinoids.

4.1.2. Lipoxygenases

5, 12, and 15 Lipoxygenases (LOX), are related to the dioxygenase family. They are involved in the polyunsaturated fatty acid metabolism in human skin. Some of these LOX are involved in skin disorders. 15-LOX was found to exist in two isoforms, 15-LOX-1 and 15-LOX-2. 15-LOX-1 was reported to be the main enzyme metabolizing linoleic acid into 13-hydroxyoctadecadienoic acid (13-HODE) (Baer et al., 1991), while 15-LOX-2 metabolize AA into 15-HETE (Brash et al., 1997). 12-LOX and 15-LOX expression in human keratinocyte cell line has been demonstrated to be affected by UV radiation. UVR decrease the 12-LOX and increase the 15-LOX levels in these cells line (Yoo et al., 2008). In addition, Rhodes et al. found that LOX metabolites such as 11-HETE, 12-HETE and 15-HETE were increased at, 4h, 18h and 24h respectively, after UVB irradiation of human skin (Rhodes et al., 2009).

12-LOX has been reported to metabolise the endocannabinoid AEA to produce the ethanolamide derivative of 12(S)-HETE-EA in rat pineal gland (Hampson et al., 1995, Ueda et al., 1995). Purified 12-LOX from porcine leukocytes also has been reported to
oxygenate AEA to produce 12(S)-HETE-EA (Hampson et al., 1995). This study has also showed that the porcine 12-LOX enzyme did not discriminate between the AEA and AA. Ueda et al. reported that the 12-LOX from porcine leukocytes and the 15-LOX-1 from rabbit reticulocytes could oxygenate AEA at rates comparable to those for AA (Ueda et al., 1995). However, human platelet 12-LOX was only marginally active, and porcine leukocyte 5-LOX was inactive with AEA as the substrate (Ueda et al., 1995). Furthermore, another study demonstrated that 12-LOX from porcine leukocytes, could efficiently oxygenate 2-AG to produce the glycerol ester of 12(S)-HETE (12(S)-HETE-G) (Moody et al., 2001). Kozak et al. showed that human 15-LOX-1, and human 15-LOX-2 metabolized 2-AG efficiently, whereas human leukocyte 5-LOXs showed no activity with this substrate (Kozak et al., 2002b).

4.2. Aims of this study

Since n-3 PUFA alter the pathways of COX and LOX enzymes that are involved in the metabolism of endocannabinoids and NAE (Kozak et al., 2002a, Kozak et al., 2002c, Kozak et al., 2004), they could have an effect on the endocannabinoids pathways in skin cells. This may lead to increased NAE production such as N-stearoylethanolamide which has been reported to have pro-apoptotic activity (Maccarrone et al., 2002) and/or N-palmitoylethanolamide that showed anti-inflammatory activity, analgesic and neuroprotective effects (Calignano et al., 1998, Lambert et al., 2002, LoVerme et al., 2005). This manipulation of the endocannabinoid pathways through n-3 PUFA could be the major defense mechanism behind the protective effect of n-3 PUFA against UVR in the skin cells. Accordingly, the hypothesis of this part of our study was based on that the n-3 PUFA particularly EPA and DHA, may play more protective role saving human skin cells from the harm of UVR through their effect on the endocannabinoids metabolizing enzymes such as COX-2 and LOXs. Therefore, this study aimed to investigate the effect of n-3 PUFA and UVR on protein levels of the inducible isoforms of COX-2, 12-LOX and 15-LOX. The effect of n-3 PUFA and UVR on the protein levels of enzymes, respectively NAPE-PLD and FAAH that control formation and degradation of endocannabinoids was also examined.
4.3 Materials and Methods

4.3.1. Western Blotting (refer to section 2.3 chapter 2)

4.3.2. Materials (refer to section 2.3.1 chapter 2)

4.3.3. Primary and secondary antibodies (refer to section 2.3.2 chapter 2)

4.3.4. Protein extraction (refer to section 2.4.1 chapter 2)

4.3.5. Determination of protein concentration (refer to section 2.4.2 chapter 2)

4.3.6. SDS-PAGE (refer to section 2.5. chapter 2)

4.3.7. Statistical analysis (refer to section 2.10. chapter 2)

4.4. RESULTS

4.4.1. The effect of UVR and n-3 PUFA on COX-2 protein levels in HaCaT keratinocytes and 46BR.IN fibroblasts.

4.4.1.1. The effect of DHA and UVR

The results shown in Figure 4.1 suggested that UVR does not significantly up-regulate COX-2 protein level in 46BR.IN fibroblasts compared to non-irradiated control cells (Figure 4.1 A and B). However, DHA treatment at concentration 10μM or 50μM significantly decreased COX-2 level (P = 0.007 and 0.002, respectively) compared to the untreated control cells (Figure 4.1 A and B). Although these effects were reversed by UVR, DHA treatments were able to retain COX-2 in lower levels compared to the non-irradiated and irradiated control cells, especially DHA 50μM which showed stronger inhibitory effect (P = 0.03) against UVR-induced COX-2 up-regulation (Figure 4.1 A and B).

The results shown in Figure 4.2 indicate some changes in COX-2 protein level among the differentially treated groups but all the below discussed changes in Figure 4.2 were not enough to be accounted as significant differences. This data indicated that COX-2 protein level was increased in UVR irradiated HaCaT keratinocytes compared to the
non-irradiated cells (Figure 4.2 A and B). In the treated HaCaT cells, COX-2 level was slightly increased in cells treated with DHA (10μM or 50μM) compared to untreated control. This effect was enhanced with UVR radiation when combined with treated cells (Figure 4.2 A and B). This data also showed that COX-2 levels were higher than any other cells when the DHA 10μM or 50μM treated cells were exposed to UV radiation.
Figure 4.1 COX-2 protein levels in DHA and UVR treated 46BR.IN fibroblasts. (A)
Western blot analysis of COX-2 protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. (B) Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the COX-2 protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the COX-2 band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the COX-2 versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. *P<0.05, **P<0.01, ***P<0.001, comparing data to control.
Figure 4.2 COX-2 protein levels in DHA and UVR treated HaCaT keratinocytes.

A. Western blot analysis of COX-2 protein levels in HaCaT keratinocytes treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the COX-2 protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the COX-2 band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the COX-2 versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
4.4.1.2. The effect of OA and UVR

The effect of OA and UVR on COX-2 protein level was followed in 46BR.IN fibroblasts untreated or treated with 10μM or 50μM OA in the presence or absence of UVR irradiation using Western blot analysis. COX-2 protein level was not found to be significantly increased after UV radiation compared to the non-irradiated 46BR.IN fibroblasts (Figure 4.3 A and B). However, treatment with OA using 10μM concentration kept COX-2 level at the same range of untreated control cells. A similar effect was seen when the OA was used with UVR compared to the irradiated control cells (Figure 4.3 A and B). Whereas, increasing the concentration of OA up to 50μM caused the COX-2 level to be slightly upregulated compared to untreated control cells. However, no change was seen when these cells were treated with UVR compared to the irradiated control cells (Figure 4.3 A and B). The same result was seen in the data shown in Figure 4.4 where the effect of OA and UVR on COX-2 protein level in HaCaT keratinocytes is depicted. These data (Figure 4.4 A and B) also indicated that the effect of 10μM or 50μM OA alone or in combination with UVR on COX-2 protein level was similar to that seen in 46BR.IN fibroblasts under the same conditions.

4.4.1.3. The effect of EPA and UVR

The effect of EPA and UVR on COX-2 protein level was investigated in 46BR.IN fibroblasts and HaCaT keratinocytes, untreated or treated with 10μM or 50μM EPA in the presence or absence of UVR irradiation using Western blot analysis. COX-2 protein level was observed to be increased in UVR irradiated cells in both 46BR.IN fibroblasts and HaCaT keratinocytes compared to their untreated control cells (Figure 4.5 A and B and Figure 4.6 A and B). Dose dependent effect appeared as a down-regulation of COX-2 protein level was recorded in 46BR.IN fibroblasts and HaCaT keratinocytes treated with EPA 10μM or 50μM compared to the untreated control cells. Nevertheless the statistical analysis did not show any significant differences (Figure 4.5 A and B and Figure 4.6 A and B). However, COX-2 protein levels were noted to be increased after
those groups of EPA treatment were submitted to UVR exposure in both cells models; 46BR.IN fibroblasts and HaCaT keratinocytes (Figure 4.5 A and B and Figure 4.6 A and B).

**Figure 4.3 COX-2 protein levels in OA and UVR treated in 46BR.IN fibroblasts.** A. Western blot analysis of COX-2 protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the COX-2 protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the COX-2 band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the COX-2 versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.4 COX-2 protein levels in OA and UVR treated in HaCaT keratinocytes. A. Western blot analysis of COX-2 protein levels in HaCaT keratinocytes treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. Data are representative of 3 experiments. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the COX-2 protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the COX-2 band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the COX-2 versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.5 COX-2 protein levels in EPA and UVR treated in 46BR.IN fibroblasts. A. Western blot analysis of COX-2 protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM EPA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. Data are representative of 3 experiments. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the COX-2 protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the COX-2 band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the COX-2 versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.6 COX-2 protein levels in EPA and UVR treated in HaCaT Keratinocytes.

A. Western blot analysis of COX-2 protein levels in HaCaT Keratinocytes treated with 10μM or 50μM EPA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the COX-2 protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the COX-2 band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the COX-2 versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
4.5. The effect of UVR and n-3 PUFA on LOX protein levels in HaCaT keratinocytes and 46BR.IN fibroblasts.

LOX mediated metabolism of AEA and 2-AG has been discussed before in this chapter (see section 4.2.1). Several Western blots experiments were carried out to assess the effect of UVR and n-3 PUFA on LOX protein in HaCaT keratinocytes and 46BR.IN fibroblasts. Since it is difficult to detect LOX protein levels possibly because of low levels of expression of this enzyme in human skin cells three representative experiments will be presented in this section.

Data in Figure 4.7 showed that 12-LOX protein level was increased after UVR exposure in the irradiated 46BR.IN fibroblasts cells compared to non-irradiated control cells. However a small decrease in its level was observed in cells treated with OA 10μM. On the other hand, in cells treated with OA 10μM plus UVR, 12-LOX level was as same as its level in the irradiated control cells (Figure 4.7 A and B). Also, cells that treated with OA 50μM and OA 50μM plus UVR showed similar level of 12-LOX to that in untreated and irradiated control cells respectively (Figure 4.7 A and B). Results in Figure 4.8 also showed that 12-LOX level was increased in the irradiated control cells compared to non-irradiated. Cells treated with DHA10μM presented minor decreases effect in 12-LOX level compared to untreated 46BR.IN fibroblasts (Figure 4.8 A and B). This effect was increased when a higher DHA concentration was used (50μM). UVR failed to increase 12-LOX level in the DHA treated (10μM or 50μM) cells (Figure 4.8 A and B). As in 46BR.IN fibroblasts, HaCaT keratinocytes cells treated with UV increased12-LOX level compared to the non-irradiated cells (Figure 4.9 A and B). No changes in 12-LOX levels were seen in cells treated with DHA 10μM or 50μM compared to untreated control cells (Figure 4.9 A and B). Combined with UVR, DHA 10μM or 50μM also did not induce any change in 12-LOX protein level (Figure 4.9 A and B).
Figure 4.7 12-LOX protein levels in OA and UVR treated in 46BR.IN fibroblasts.

A. Western blot analysis of 12-LOX protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated \((n = 1)\). GAPDH was used as loading control. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the 12-LOX protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the 12-LOX band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the 12-LOX versus the intensity of the GAPDH band in the other groups was calculated accordingly.
Figure 4.8 12-LOX protein levels in DHA and UVR treated in 46BR.IN fibroblasts.

A. Western blot analysis of 12-LOX protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated (n = 1). GAPDH was used as loading control. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the 12-LOX protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the 12-LOX band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the 12-LOX versus the intensity of the GAPDH band in the other groups was calculated accordingly.
Figure 4.9 12-LOX protein levels in OA and UVR treated in HaCaT keratinocytes.

A. Western blot analysis of 12-LOX protein levels in HaCaT keratinocytes treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated (n = 1). GAPDH was used as loading control. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the 12-LOX protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the 12-LOX band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the 12-LOX versus the intensity of the GAPDH band in the other groups was calculated accordingly.
4.6. The effect of UVR and n-3 PUFA on NAPE-PLD and FAAH protein levels in HaCaT keratinocytes and 46BR.IN fibroblasts

NAPE-PLD and FAAH are the enzymes responsible for endocannabinoid biosynthesis and degradation, respectively. Protein levels of these enzymes were investigated in HaCaT keratinocytes and 46BR.IN fibroblasts. In three independent experiments, these cells were treated with DHA, EPA and OA at two doses: 10μM or 50μM. Each experiment was divided into two groups; control with and without UVR and n-3 PUFAs treated with and without UVR (see sections 2.2.5 and 2.2.6 for more details). NAPE-PLD and FAAH protein levels were estimated by Western blot analysis.

4.6.1. Effect of OA and UVR on FAAH and NAPE-PLD in HaCaT keratinocytes

The effect of OA and UVR on FAAH protein level in HaCaT keratinocyte cell line was assessed using Western blot analysis (Figure 4.10 A). Insignificant down-regulation effect was seen in FAAH protein level in the irradiated compared to non-irradiated HaCaT cells (Figure 4.10 A and B). Data in this figure showed that OA treatment at dose 10μM or 50μM increased FAAH levels compared to the untreated control cells (Figure 4.10 A and B). However, a combination of OA 10μM or 50μM treated groups with UVR did not affect FAAH levels significantly compared to the irradiated controls cells (Figure 4.10 A and B). NAPE-PLD levels were slightly increased after UVR exposure compared to the non-irradiated control cells. Furthermore, treatment either with OA 10μM or 50μM insignificantly decreased NAPE-PLD compared to the non-irradiated. Moreover, NAPE-PLD levels also were insignificantly reduced with OA 10μM or 50μM plus UVR compared to the irradiated control cells. In general, NAPE-PLD protein levels were almost similar in all treated groups either with or without UV radiation (Figure 4.11 A and B).
4.6.2. Effect of EPA and UVR on FAAH and NAPE-PLD in HaCaT keratinocytes cell lines.

FAAH protein level was reduced in the irradiated control HaCaT keratinocytes compared to the non-irradiated control cells (Figure 4.12 A and B). Dose dependently increase was observed in HaCaT keratinocytes treated with EPA 10µM or 50µM compared to untreated HaCaT cells (Figure 4.12 A and B). However, this effect was reversed by UVR in both, EPA10µM and 50µM treated groups. In this case FAAH levels were reduced to the same level as in the irradiated control cells (Figure 4.12 A and B).

Data presented in Figure 4.13 suggested that UV radiation increased NAPE-PLD protein level in HaCaT cells compared to the non-irradiated control. Furthermore, NAPE-PLD protein levels also increased in cells treated with EPA 10µM and this effect was enhanced with EPA 50µM (P = 0.002) compare to the untreated HaCaT cells (Figure 4.13 A and B). However, combination of EPA 10µM and UVR induced insignificant decreases in NAPE-PLD level compared to the irradiated control cells but this effect was reversed in cells treated with EPA 50µM plus UVR compared to the irradiated control HaCaT cells (Figure 4.13 A and B).

4.6.3. Effect of DHA and UVR on FAAH and NAPE-PLD in HaCaT keratinocytes

Data in Figure 4.14 indicated that FAAH protein level was reduced after HaCaT cells were submitted to UV radiation compared to the non-irradiated control cells. However, this level was increased in cells treated with treated with DHA 10µM compared to untreated cells (Figure 4.14 A and B). This effect was then prevented by UVR which reduced FAAH levels but to range upper than in the irradiated control cells. Likewise, treatment with DHA 50µM decreased FAAH to its lowest level in this experiment and this effect was however terminated by UV radiation which brought FAAH level to be as same as in the irradiated cells treated with DHA 10µM (Figure 4.14 A and B). NAPE-PLD protein level was decreased in HaCaT keratinocytes submitted to UVR compared to non-irradiated control cells (Figure 4.15 A and B). Furthermore, its level was also decreased in cells treated with DHA10µM or 50µM compared to untreated cells.
However, when this group was exposed to UVR these effects were reversed (Figure 4.15 A and B).

**Figure 4.10: FAAH protein levels in OA and UVR treated in HaCaT keratinocytes.**

**A.** Western blot analysis of FAAH protein levels in HaCaT keratinocytes treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. **B.** Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the FAAH protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the FAAH band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the FAAH versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.11: NAPE-PLD protein levels in OA and UVR treated in HaCaT keratinocytes. A. Western blot analysis of NAPE-PLD protein levels in HaCaT keratinocytes treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the NAPE-PLD protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the NAPE-PLD band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the NAPE-PLD versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.12: FAAH protein levels in EPA and UVR treated in HaCaT keratinocytes.

A. Western blot analysis of FAAH protein levels in HaCaT keratinocytes treated with 10μM or 50μM EPA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown.

B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the FAAH protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the FAAH band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the FAAH versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.13: NAPE-PLD protein levels in EPA and UVR treated in HaCaT keratinocytes. A. Western blot analysis of NAPE-PLD protein levels in HaCaT keratinocytes treated with 10μM or 50μM EPA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the NAPE-PLD protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the NAPE-PLD band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the NAPE-PLD versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. **P<0.01, comparing data to control.
Figure 4.14: FAAH protein levels in DHA and UVR treated in HaCaT keratinocytes.

A. Western blot analysis of FAAH protein levels in HaCaT keratinocytes treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown.

B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the FAAH protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the FAAH band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the FAAH versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.15: NAPE-PLD protein levels in DHA and UVR treated in HaCaT keratinocytes. A. Western blot analysis of NAPE-PLD protein levels in HaCaT keratinocytes treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the NAPE-PLD protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the NAPE-PLD band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the NAPE-PLD versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
4.6.4. Effect of OA and UVR on FAAH and NAPE-PLD protein level in 46BR.IN fibroblasts

The effect of OA and UVR on FAAH protein level in 46BR.IN fibroblasts was studied using western blot analysis. Our findings demonstrated that there were no significant changes in FAAH protein level in UVR irradiated 46BR.IN cells compared to non-irradiated control cells (Figure 4.16 A and B). However, no changes were observed in FAAH levels in OA 10μM or 50μM treated cells compared to the untreated control cells. Compared to the irradiated control cells, minor increases were noticed in FAAH levels when those treated cells exposed to UVR.

NAPE-PLD protein level showed similar patterns to that of FAAH protein levels in 46BR.IN fibroblasts where its level was also insignificantly decreased after UVR exposure compared to non-irradiated control cells. This minor effect was enhanced when the OA (10μM or 50μM) treatment was combined with UV radiation (Figure 4.17 A and B). Data also showed a slight reduction in NAPE-PLD level in cells treated with OA 10μM. In contrast, OA at concentration 50μM caused the level of NAPE-PLD to be increased compared to untreated control cells (Figure 4.17 A and B).

4.6.5. Effect of EPA and UVR on FAAH and NAPE-PLD in 46BR.IN fibroblasts

FAAH protein level was reduced after UV radiation compared to the non-irradiated control cells. EPA treatment in both doses used (10μM and 50μM) also induced minor decrease in FAAH level compared to the untreated cells (Figure 4.18 A and B). A similar effect was observed when those treated cells were submitted to UV radiation (Figure 4.18 A and B).

The data shown in Figure 4.19 showed that there were minor increases in NAPE-PLD levels in the irradiated cells compared to non-irradiated control cells (Figure 4.19 A and B). In addition, both doses used (10μM or 50μM) of EPA induced dose dependently minor increases compared to the untreated cells (Figure 4.19 A and B). This effect was enhanced when those treated cells were exposed to UV radiation.
4.6.6. Effect of DHA and UVR on FAAH and NAPE-PLD in 46BR.IN fibroblasts

FAAH protein levels were found to be slightly decreased in the irradiated 46BR.IN fibroblasts compared to the non-irradiated control cells (Figure 4.20 A and B). Also, a similar effect was observed in DHA 10µM or 50µM and in DHA 50µM plus UVR (Figure 4.20 A and B). However, a small increase in FAAH level was seen in cells treated with DHA 10µM combined with UV radiation (Figure 4.20 A and B). Data in Figure 4.21 indicated that NAPE-PLD protein level also was reduced after UVR compared to non-irradiated cells (Figure 4.21 A and B). Comparing to the untreated control cells, DHA 10µM also showed the same effect. On the other hand, treatment with DHA 50µM and DHA 10 µM or 50 µM plus UVR increased NAPE-PLD levels to the same range compared to the irradiated and non-irradiated control cells (Figure 4.21 A and B).
**Figure 4.16: FAAH protein levels in OA and UVR treated 46BR.IN fibroblasts.**

A. Western blot analysis of FAAH protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the FAAH protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the FAAH band versus the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the FAAH versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.17: NAPE-PLD protein levels in OA and UVR treated in 46BR.IN fibroblasts. A. Western blot analysis of NAPE-PLD protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM OA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the NAPE-PLD protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the NAPE-PLD band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the NAPE-PLD versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.18: FAAH protein levels in EPA and UVR treated in 46BR.IN fibroblasts.

A. Western blot analysis of FAAH protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM EPA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown.

B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the FAAH protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the FAAH band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the FAAH versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.19: NAPE-PLD protein levels in EPA and UVR treated in 46BR.IN fibroblasts. A. Western blot analysis of NAPE-PLD protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM EPA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the NAPE-PLD protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the NAPE-PLD band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the NAPE-PLD versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.20: FAAH protein levels in DHA and UVR treated in 46BR.IN fibroblasts.

A. Western blot analysis of FAAH protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown.

B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the FAAH protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the FAAH band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the FAAH versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
Figure 4.21: NAPE-PLD protein levels in DHA and UVR treated in 46BR.IN fibroblasts. A. Western blot analysis of NAPE-PLD protein levels in 46BR.IN fibroblasts treated with 10μM or 50μM DHA and irradiated with UVR or left untreated as indicated. GAPDH was used as loading control. One representative out of 3 independent experiments is shown. B. Densitometry analysis of Western blots was carried out using ImageJ software. The intensity of the NAPE-PLD protein band was normalized to that of the GAPDH protein band. The ratio of the intensity of the NAPE-PLD band with the intensity of the GAPDH band in the non-treated cells was arbitrarily set to 1. The ratio of the intensity of the NAPE-PLD versus the intensity of the GAPDH band in the other groups was calculated accordingly. Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
4.7 Discussion

4.7.1. The effect of omega-3 polyunsaturated fatty acids and UVR on COX-2 protein expression in HaCaT keratinocytes and 46BR.IN fibroblasts

UVR radiation can cause skin disorders like sunburn, DNA damage and carcinogenesis. It is well documented that COX-2 overexpression post UVR is involved in skin inflammation and skin cancer and it may be the key factor behind all the detrimental effects that UVR induces. However, n-3 PUFA are well known for their beneficial role as anti-inflammatory compounds which may be a promising component that may play a crucial role against the detrimental effects of UVR on skin cells. Therefore, in this study we sought to examine the effect of n-3 PUFA on UVR-induced up-regulation in COX-2 protein expression in HaCaT keratinocytes and 46BR.IN fibroblasts.

COX-2 is expressed in human keratinocytes and fibroblasts and involved in keratinocyte differentiation (Leong et al., 1996) and human foreskin fibroblastic (HFF) cells (Gilroy et al., 2001). Many factors may be involved in COX-2 expression such as multiple mitogenic and pro-inflammatory stimuli (Maldve and Fischer, 1996, Feng et al., 1995). Among these factors, growth factors (Feng et al., 1995), cytokines (Feng et al., 1995), bacterial endotoxin (Xie et al., 1992) and carcinogens (Kujubu et al., 1991). Also, as mentioned before during the differentiation process in human keratinocytes (Leong et al., 1996) or fibroblastic cells (Gilroy et al., 2001). According to the Gilroy study, COX-2 expression is increased in fibroblasts cells when they were exposed to phorbol 12-myristate 13-acetate (PMA) or interleukin-1β (IL-1β), this happened during the quiescent G0 phase. Whereas when the cells entered the committed G1 phase of the cell cycle, COX-2 expression was suppressed. Also it was reported that COX-2 mRNA levels were reduced by 53% and 70% when a majority of the cells had entered the S and G2/M phases of cell cycle, respectively. Gilroy et al., also suggested that COX-2 expression in cycling cells is firmly controlled to avoid excessive oxidative DNA damage. Therefore, any dysregulation in this system could lead to detrimental effects.

Results from our study showed that COX-2 levels were up-regulated in the UVR irradiated cells in levels slightly higher than those that were not irradiated in both cell
models. These results are in agreement with other previous studies showing up-regulation of COX-2 protein levels following UVB irradiation in HaCaT keratinocytes (Buckman et al., 1998, Tang et al., 2001, Kim et al., 2007). COX-2 protein expression was also found to be up-regulated in human keratinocytes in volunteers subjected to UVB radiation up to 4 times (Buckman et al., 1998). COX-2 was also overexpressed in the skin of hairless mice upon UVB radiation (Kim et al., 2007). Moreover, Bachelor et al., reported an increase in COX-2 mRNA after UVB radiation in SKH-1 mouse epidermis (Bachelor et al., 2005). Findings from all of the above mentioned studies were in agreement with our results.

It has been suggested that UVB induces COX-2 expression throughout increased activation of transcription factors, p38 mitogen activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K) which will then phosphorylate the cyclic AMP-responsive element binding protein (CREB) which is needed for the COX-2 to be expressed (Bachelor et al., 2005). In addition, Tang et al., (2001) demonstrated that CREB is essential for both basal and UVB induced COX-2 expression in HaCaT keratinocytes (Tang et al., 2001). Furthermore, NF-kB is another transcription factor which may play a key role in the UVB-induced COX-2 expression. Accordingly, UVB has been found to lead to phosphorylation of ERK (Chang et al., 2011) and p38 mitogen-activated kinases (Bachelor et al., 2005, Kim et al., 2005) which then activates NF-kB and induces COX-2 expression (Chun and Surh, 2004). However, Chen et al., demonstrated that the ERK was not essential for UVB-induced COX-2 gene expression in HaCaT keratinocytes (Chen et al., 2001). Moreover, it has been suggested that UVR induced cytoplasmic expression of human antigen R (HuR) which in turn increases COX-2 mRNA stability (Sengupta et al., 2003, Tong et al., 2007).

The effect of the n-3 PUFA DHA and EPA on COX-2 expression was also examined. Our findings indicated that DHA treatment significantly decreased COX-2 expression in 46BR.IN fibroblasts in dose dependent manner (Figure 4.1). UVR-induced COX-2 expression was also dose dependently inhibited by 10μM or 50μM DHA in the same cells. Similar results in different cell lines have been demonstrated by previous study where DHA reported to block COX-2 expression in UVR stimulated keratinocytes
(Zhang and Bowden, 2008). Moreover, DHA has been also shown to revert UVR-induced COX-2 expression in nr-HaCaT cells (Sengupta et al., 2003, Zhang and Bowden, 2008). In this case DHA reduced the expression of COX-2 mRNA stabilizer HuR in many cells including keratinocytes (Sengupta et al., 2003, Zhang and Bowden, 2008). Moreover, topical application of DHA has been also reported to inhibit UVB-induced COX-2 expression in mouse skin (Rahman et al., 2011). According to Rahman et al., (2011) this effect was attributed to the ability of DHA to attenuate UVB-induced DNA binding of NF-kB through the inhibition of the phosphorylation of IKKα/β and phosphorylation and degradation of IKBα and nuclear localization of p50 and p65 proteins. However, our results showed an opposite effect, when HaCaT keratinocytes were treated with DHA 10µM or 50µM, DHA did not inhibit UVR induced COX-2 expression in the irradiated cells (Figure 4.2). Our results here are also in consistent with another study that used a different stimulus and different type of cells. They were found that the DHA enhanced phorbol ester or interleukin 1β-induced COX-2 expression in rat vascular endothelial cells (Machida et al., 2005). The authors attributed this effect to the ability of DHA induced p44/42 MAPK activation. Similar effect of DHA was also reported by Hirafuji et al., and Diep et al., who suggested the role of DHA induced p44/42 or p38 MAPK activation (Diep et al., 2000, Hirafuji et al., 2002).

Data from the present study also showed that EPA 10µM or 50µM decreased COX-2 protein levels in concentration dependent manner in both HaCaT keratinocytes and 46BR.IN fibroblasts cells. These results are in line with what another study indicated that COX-2 expression was increased in young skin human after EPA treatment (Kim et al., 2006). EPA and DHA also reduced COX-2 expression in HT-29 human colorectal cells (Calviello et al., 2004). Furthermore, COX-2 expression was found to be decreased in rats treated with flaxseed oil which is a high source of n-3 PUFA (Bommareddy et al., 2006). Similar to DHA, EPA may exert its effect on COX-2 expression through NFkB. EPA has been reported to inhibit NFkB expression in cultured pancreatic and human monocyes via inhibition of IkB degradation, the inhibitory subunit of NFkB (Lo et al., 1999, Novak et al., 2003, Zhao et al., 2004). This finding has been shown by another study that suggested that endogenously biosynthesized n-3 PUFA protect transgenic
mice from colitis through a decrease in NFkB activity (Hudert et al., 2006). However, our results showed that both EPA treatment and UV radiation synergistically induced COX-2 expression in both cells types. Our data also showed that UVR significantly increased COX-2 level in EPA treated HaCaT keratinocytes. In another study, a synergistic action has also been reported between UVB and EPA to induce TNF-α expression (Pupe et al., 2002). Indeed, TNF-α has been suggested to stimulate gene-6 (TSG-6) which in turn up-regulate COX-2 expression in macrophages which preferentially produce PGD₂ and its metabolite 15-deoxy-12,14-prostaglandinJ₂ (15dPGJ₂) (Mindrescu et al., 2005). 15dPGJ2 and n-3 PUFA both may play a key role in the activation of the anti-inflammatory peroxisome-proliferator-activated-receptor gamma (PPAR gamma) (Jump and Clarke, 1999, Mindrescu et al., 2005). In addition, EPA and DHA has also been found to up-regulate COX-2 expression in HaCaT keratinocytes (Chene et al., 2007). The effect could be essential to increase the production of 3-series PGs (i.e. PGD3, PGI3 and PGE3) through EPA and DHA-induced COX-2 expression (Fischer et al., 1988). It should be noted that the 3-series PGs may play an important role as an anti-inflammatory eicosanoids as they are weaker inflammatory than 2-series PGS (Bagga et al., 2003).

4.7.2. The effect of omega-3 polyunsaturated fatty acids and UVR on NAPE-PLD and FAAH protein expression in HaCaT keratinocytes and 46BR.IN fibroblasts

Here we explore for the first time the effect of the UVB on the NAPE-PLD and FAAH, the two enzymes that responsible for AEA biosynthesis and degradation respectively. Although, there were no significant differences, our results showed some trends indicating that UVR may reduce FAAH levels while, mixed effects observed on NAPE-PLD levels in HaCaT keratinocytes and 46BR.IN fibroblasts. These were supported by other observations presented in this study that AEA and 2-AG synthesis was found to be slightly increased after UVR exposure of both types of cells models used in this study (Figures 3.2 F, 3.7 F, 3.8 F and 3.9 F: Chapter 3).
DHA and EPA treatment decreased FAAH levels and increased NAPE-PLD levels in both HaCaT keratinocytes and 46BR.IN fibroblasts. This has lent support to other results in this study showing that DHA increased AEA production in HaCaT keratinocytes (Figure 3.2 F. Chapter 3).

In an attempt to explain our findings, we examined many aspects. Presumably, UVR could affect FAAH and NAPE-PLD protein levels through their transcriptional factors and gene expression. NF-kB is a transcription factor that plays a key role in cellular response to environmental stress such as UV radiation by inducing or suppressing the expression of specific gene (Herrlich et al., 2008). UVR-induced NF-kB activation has been reported in several studies. For example, NF-kB activation in human skin fibroblasts has been demonstrated after UVA radiation (Vile et al., 1995, Reelfs et al., 2004). Also, UVB was found to induce NF-kB activation in human epidermal keratinocytes (Adhami et al., 2003). Apart from this, Leptin was demonstrated to stimulate the activity and expression of FAAH throughout FAAH gene by activating the promoter region through a STAT3 (signal transduction and activator of transcription 3) element (Maccarrone et al., 2003a). Moreover, Waleh et al., has reported that FAAH enzyme has estrogen response element (ERE) in its FAAH gene in mouse (Waleh et al., 2002). Thus, sex hormones have been reported to down-regulate FAAH gene expression (Maccarrone et al., 2000). Progesterone for example was found to up-regulate the FAAH gene in human T-cells via the Ikaros transcription factor (Maccarrone et al., 2003a). In contrast, endogenous steroid 17β-estradiol (E2) has been suggested to regulate many transcription factors and may be enhance FAAH gene expression at transcriptional and translational level (Rossi et al., 2007). It is noteworthy noting that estrogen receptors (ER) has been found to interact with some transcription factors to regulate or repress transcription (Cheng et al., 2003). NF-kB is one of these transcription factors that were found to interact with ER (Frasor et al., 2009). Therefore, an interaction between NF-kB and ER may be one of the mechanisms behind UVR-induced FAAH down-regulation observed in our results. This is in line with previous observation that suggested NF-kB could regulate FAAH expression (Maccarrone et al., 2003b).
Furthermore, Liu et al., has suggested NF-kB as the main transcription factor that regulates LPS-induced AEA synthesis (Liu et al., 2003).

Data from the present study also showed that UV radiation affected NAPE-PLD protein levels in a fluctuated manner. However, the general trend was that UVR suppressed NAPE-PLD expression. These data are in line with another research reported that LPS produce an inhibition effect on NAPE-PLD expression (Zhu et al., 2011). In fact, these observations may be due to many reasons, the effect of UV radiation on gene expression of this enzyme could be one of them. NAPE-PLD gene was cloned in rodent and human (Okamoto et al., 2004). The expression of this gene has been found to be controlled by the transcription factor SP1 (Zhu et al., 2011). An environmental stress such as UV radiation may affect gene expression through up- or downregulation of their transcriptional factors. These may occur via the interaction of transcriptional factors with each other as mentioned earlier. For instance, a functional interaction between SP1 and NF-kB has been shown to induce IL-6 gene expression (Sancéau et al., 1995). Also, p65 which is subunit of NF-kB has been found to synergistically activate HIV-1 transcription with SP1 (Perkins et al., 1994, Pazin et al., 1996). Therefore, interaction of NF-kB with SP1 can act synergistically to activate transcription factor of many target genes. Therefore, this is may be the case behind UVR-induced changes in NAPE-PLD levels in our study, especially with the notion of that the environmental stress induced genes expression was found to be controlled by the transcription factor, NF-kB (Hayden and Ghosh, 2012).

NF-kB itself could be controlled by n-3 PUFA which then regulates cellular signalling and genes expression, in particular those related to inflammatory signalling and lipid metabolism (Sampath and Ntambi, 2005, Schmitz and Ecker, 2008). In addition, n-3 PUFA could affect the membrane phospholipids and as a result, the production of eicosanoids (Sampath and Ntambi, 2005). As an example, n-3 PUFA have been found to affect eicosanoids production through their inhibitory effect on leptin and then the expression of lipogenic gene in adipose tissue (Jump and Clarke, 1999, Raclot et al., 1997). This may explain the down-regulation of FAAH by EPA and DHA observed in our study, as leptin was found to stimulate the activity of FAAH gene expression.
(Maccarrone et al., 2003a). As a matter of interest, the effect of n-3 PUFA on genes expression seems to be very complicated. For example, they could exert positive and negative effects on NF-kB (Sampath and Ntambi, 2005). n-3 PUFA can initiate the phosphorylation of the IkB and then allow NF-kB nucleus translocation and as a results the transcription of different genes such as COX-2 and IL-6 (Calder, 1997, Khalfoun et al., 1997). In contrast, n-3 PUFA was reported to decrease the phosphorylation of the IkB and then inhibit NF-kB translocation and target gene transcription (Camandola et al., 1996, Zhao et al., 2004). All these could explain the effects of n-3 PUFA on the FAAH and NAPE-PLD protein levels observed in the current study.

Taken together these results, it suggest that n-3 PUFA can induce endocannabinoid production through their effect on endocannabinoid related enzymes. UVR-induced endocannabinoid biosynthesis could suggest a defense mechanism of skin cells followed UV radiation. However, further studies are still required.
CHAPTER 5: Investigation the effect of ultraviolet radiation on endocannabinoids and N-acylethanolamines in White Caucasians of skin phenotype II and South Asians of skin phenotype V

5.1. Introduction

The endocannabinoids AEA and 2-AG and NAE are widely distributed in many cells and tissues (Devane et al., 1988, Devane et al., 1992, Matsuda et al., 1990, Mechoulam et al., 1995, Schmid, 2000). They are involved in many physiological and pathological functions including anti-nociception, anti-inflammatory effects, reproduction, modulation of vascular tone, obesity, cancer, schizophrenia, sleep and drug addiction (Di Marzo, 1998, Piomelli, 2003, Pacher et al., 2006).

Ultraviolet radiation (UVR) is an environmental insult that can cause several health disorders to human beings ranging from skin inflammation to skin cancer. It is well known that UVR exerts its harmful effects on the human body through their interaction with many biological processes such as genes expression and lipid biosynthesis and metabolism pathways. Endocannabinoids and NAE have been suggested to play a key role as anti-inflammatory and anti-cancer bioactive lipids. However, the effect of UVR on their biochemical pathways has not been fully studied. The effect of UVR on endocannabinoids and their congeners on human skin cells was examined in chapters 3 and 4 of this study. Here we examine the hypothesis that UVR may affect endocannabinoid levels in the circulation and we examined this using volunteers of two different ethnicities. Therefore, the specific objective here was to investigate the effect of UVR on serum endocannabinoids and NAE. Volunteers of two skin types were used to measure the levels of serum endocannabinoids and NAE in response to multiple doses of UVR.
5.2. Aim of the study

To explore the effect of UVR on the levels of endocannabinoids and N-acylethanolamines in human serum from White Caucasians of skin phototype II and South Asians of phototype V.

5.3. Materials and Methods (For more details about the volunteers, experimental design and lipid extraction please refer to Chapter 2, sections 2.9.1, 2.9.2, 2.9.3, 2.9.4, 2.9.5 and 2.9.6).

5.3.1. Statistical Analysis (refer to chapter 2 section 2.10).

5.4. RESULTS

Volunteers were from two different ethnic groups; 10 White Caucasians of skin phototype II and 6 South Asians of skin phototype V. They were subjected to 8 doses of UVR each time receiving the same dose (1.3 SED) from day 4, 7, 14, 21, 28, 35 and day 39 of the study, the baseline was coded as day 0.

5.4.1. Effect of UVR on serum endocannabinoids and N-acylethanolamines

5.4.1.1 Effect of UVR on serum palmitoylethanolamide

Palmitoylethanolamide (PEA) level in White Caucasians was estimated to be 3160 pg/ml on day 0 which represents the baseline of the PEA, pre UVR. On day 4, PEA level was increased to 4048 pg/ml and then from the day 7 to day 39, PEA levels were almost as same as the baseline level (Figure 5.1, compare day 0 to days 7, 14, 21, 28, 35 and 39 ). PEA baseline level in South Asians participants was found to be 2814 pg/ml on day 0. Compared to this baseline, PEA levels were slightly decreased to 2571 pg/ml, 2473 pg/ml, 2533 pg/ml and 2474 pg/ml on day 4, 7, 21 and 39 respectively. Whereas, on day 28 which is the time point for the fifth dose of the UVR, PEA level was slightly increased up to 3023 pg/ml compared to the baseline (Figure 5.1, compare day 0 to day 28). While, on day 14 and 35 the PEA levels were approximately similar to the baseline level (Figure 5.1, compare day 0 to day 14 and 35). Overall, One-Way ANOVA followed
by Tukey post hoc test did not show any significant differences in PEA levels between the different time points of UVR exposure either in the White Caucasians of skin phototype II or in the South Asians of skin phototype V. As a result, it was concluded that exposure to UVR for 6 weeks did not cause significant changes in PEA levels in the human serum samples from White Caucasians of skin phototype II and South Asians of skin phototype V.

**Figure 5.1.** PEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.2. Effect of UVR on serum linoleoyalethanolamide

Linoleoyalethanolamide (LEA) baseline levels were measured to be 870pg/ml and 1015pg/ml in skin phototype II and skin phototype V respectively. The comparison between the different time points of UVR exposure was done by One-way ANOVA following by Tukey Post hoc tests. There were no significant changes in LEA levels in both skin phototype between all-time points of UVR exposures. LEA baseline in White Caucasians of skin phototype II was assessed to be 870pg/ml. UVR exposure from day 4 to day 21 did not induce any noteworthy changes in LEA levels compared to the baseline on day 0. However, in day 28 LEA level was increased up to 1004 pg/ml and then decreased again to 947 pg/ml, 885 pg/ml in day 35 and 39 respectively. In addition, the LEA baseline in South Asians of skin phototype V was calculated to be 1015 pg/ml. The repeated doses of UVR on days 4, 7, 21 and 39 decreased the LEA level to 916 pg/ml, 920 pg/ml, 928 pg/ml and 969 pg/ml respectively. However, on day 14, 28 and 35 there were no important changes in LEA level compared to the baseline (Figure 5.2, compare day 0 to day 14, 28 and 35). Consequently, One-Way ANOVA showed that 6 weeks of UVR exposure did not bring about any significant changes in LEA levels between the different time points in the examined groups.

Figure 5.2. LEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.3. Effect of UVR on serum oleylethanolamide

OEA baseline levels were calculated to be 1412pg/ml and 798pg/ml in skin phototype II and skin phototype V respectively. The comparison between the OEA levels in each time points of UVR exposure was done by One-way ANOVA following by Tukey Post hoc tests. Accordingly, there were no significant changes in OEA levels either in skin phototype II or phototype V (Figure 5.3).

OEA baseline level in White Caucasi ans of skin phototype II was calculated to be 1412pg/ml while, this level slightly increased to 1513pg/ml, 1700pg/ml and 1589pg/ml on day 4, day 28 and day 39 respectively. However, there were no intense changes in OEA levels among the other time points of UVR exposure representative on day 7, 14, 21 and 35 (Figure 5.3). In South Asians of phototype V, OEA baseline level was estimated to be 798pg/ml on day 0. Compared to this baseline level, no significant changes were seen in OEA level especially on day 4, 14 and 28 (Figure 5.3, compare day 0 to day 4, 14 and 28). However, on day 7, 21, 35 and 39 OEA levels insignificantly decreased to 684pg/ml, 598pg/ml, 675pg/ml and 681pg/ml respectively (Figure 5.3, compare day 0 to day 7, 21, 35 and 39).

**Figure 5.3.** OEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.4. Effect of UVR on serum stearoylethanolamide

Stearoylethanolamide (STEA) baseline levels were considered to be 713 pg/ml and 671 pg/ml in skin phototype II and skin phototype V respectively. The comparison between the STEA levels in each time points of UVR exposure was done by One-way ANOVA following by Tukey post hoc test. As a result, there were no significant changes in STEA levels in both skin phototype between all-time points of UVR exposures. STEA baseline level in the White Caucasians group was estimated to be 713 pg/ml (Figure 5.4). However, STEA level was 943 pg/ml after the first dose of the UVR on day 4. In the South Asians group STEA baseline was assessed to be 671 pg/ml (Figure 5.4). However, STEA levels in volunteers from South Asia were decreased on day 4 and 7 after the first two doses of the UVR to 436 pg/ml and 457 pg/ml respectively. Whereas, on day 14, 21, 28, 35 and 39, STEA levels were increased to 548 pg/ml, 493 pg/ml, 591 pg/ml, 560 pg/ml and 557 pg/ml respectively (Figure 5.4, compare day 0 to days 4, 7, 14, 21, 28, 35 and 39). Generally, different time points of UVR exposure did not induce any significant changes in STEA levels in both tested groups.

Figure 5.4. STEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.5. Effect of UVR on serum docosahexaenoyl ethanolamide

Docosahexaenoyl ethanolamide (DHEA) baseline levels were considered to be 2797 pg/ml and 792 pg/ml in skin phototype II and skin phototype V respectively. The comparison between the DHEA levels in each time points of UVR exposure was done by One-way ANOVA following by Tukey Post hoc tests. There were no significant differences in DHEA levels of both tested groups of skin phototypes (Figure 5.5). DHEA baseline level was considered to be 2797 pg/ml (day 0). Compared to this baseline, DHEA level was decreased to 2282 pg/ml after the first dose of UVR on day 4 (Figure 5.5, compare day 0 to day 4). However, no significant differences were recorded between these time points. Regardless of DHEA levels on day 4, there were no dramatic changes in the DHEA levels between the other time points representative on day 7, 14, 21, 28, 35 and 39 (Figure 5.5). In addition, Serum samples from six South Asians of skin phototype V were extracted and analyzed for DHEA. Baseline levels were assessed to be 792 pg/ml (day 0). DHEA levels were increased to 849 pg/ml, 1081 pg/ml, 1103 pg/ml and 1127 pg/ml on days 4, 14, 28 and 35 respectively. (Figure 5.17, compare day 0 to days 4, 14, 28 and 35). However, on days 7, 21 and 39 DHEA levels were decreased to 707 pg/ml, 626 pg/ml and 755 pg/ml respectively, compared to the baseline level on day 0 (Figure 5.17, compare day 0 to days 7, 21 and 39). Accordingly, we can conclude that UVR exposure for 6 weeks did not end in any significant changes in DHEA levels in the White Caucasians and South Asians groups when the UVR effect was examined in each group separately.
Figure 5.5. DHEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.

5.4.1.6. Effect of UVR on serum arachidonoylethanolamide

Arachidonoylethanolamide (AEA) baseline levels were considered to be 326 pg/ml and 280 pg/ml in skin phototype II and skin phototype V respectively. The comparison between the AEA levels in each time points of UVR exposure was done by One-way ANOVA following by Tukey post hoc test (Figure 5.6). White Caucasians volunteers were extracted and analyzed for AEA. AEA level was estimated to be 326 pg/ml (day 0, Figure 5.6). In general, AEA levels were similar between all the time points of UVR exposure compared to the baseline on day 0. (Figure 5.6, compare day 0 to day 4, 7, 14, 21, 28, 35 and 39). Thus, no significant differences in AEA levels were found in this experiment. AEA baseline level in South Asians volunteers was considered to be 280 pg/ml (day 0, Figure 5.6). AEA levels were decreased to 150 pg/ml, 196 pg/ml and 145 pg/ml on days 4, 7 and day 21 respectively, compared to day 0 (Figure 5.6, compare
day 0 to days 4, 7 and 21). In addition, AEA levels were also slightly decreased to 272pg/ml, 202pg/ml and 237pg/ml on days 14, 35 and 39 respectively. On the other hand, on day 28 AEA level was increased to 322pg/ml compared to the baseline level (Figure 5.6, compare day 0 to day 28). At the end, no significant differences were found in this experiment.

![AEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.](image)

**Figure 5.6.** AEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.

### 5.4.1.7. Effect of UVR on serum 2-arachidonoyl glycerol

2-arachidonoyl glycerol (2-AG) baseline levels were considered to be 993 pg/ml and 1786 pg/ml in skin phototype II and skin phototype V respectively. The comparison between the 2-AG levels in each time points of UVR exposure was done by One-way ANOVA following by Tukey Post hoc tests. Based on our data, there were no significant differences in 2-AG levels in all time points. 2-AG baseline in White Caucasians group was calculated to be 993 pg/ml on day 0 (Figure 5.7). Compared to its baseline, 2-AG level was markedly increased up to 2364 pg/ml after the first exposure of UVR on day
4. In day 7, 2-AG level was decreased to 1101 pg/ml recording the nearest level to the baseline on day 0 (Figure 5.7, compare day 0 to day 7). After that, 2-AG level was increased again to 1732 pg/ml, 1575 pg/ml, 1474 pg/ml, and 1527 pg/ml on day 14, 21, 28 and 35 respectively. However, on day 39, 2-AG level decreased to 1231 pg/ml but still more than its baseline level.

2-AG baseline in South Asians group was considered to be 1786 pg/ml on day 0 (Figure 5.7). Compared to its baseline, 2-AG level was increased up to 2088 pg/ml, 2487 pg/ml and 2560 pg/ml on day 4, 21 and 28 respectively (Figure 5.7, compare day 0 to days 4, 21 and 28). Conversely, on day 7, 14, 35 and 39, 2-AG levels was slightly decreased to 1800 pg/ml, 1699 pg/ml, 1682 pg/ml and 1185 pg/ml respectively, compared to the baseline level (Figure 5.7, compare day 0 to days 7, 14, 35 and 39). Finally, our results did not show any significant changes in 2-AG levels between the different time points of UVR exposure either in White Caucasians or South Asians group.

Figure 5.7. 2-AG levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.8. Effect of UVR on serum 1-arachidonoyl glycerol

1-arachidonoyl glycerol (1-AG) baseline in White Caucasians was considered to be 742 pg/ml on day 0 (Figure 5.8). Compared to the baseline, UVR did not alter the 1-AG levels in day 4, 7, 14, 28 and 35. Whereas, 1-AG levels were slightly decreased on day 22 compared to day 0 (Figure 5.8, compare day 0 to days 4, 7, 14, 21, 28 and 35). The highest level of 1-AG was seen on day 39 (865pg/ml) (Figure 5.8, compare day 0 to day 39). 1-AG baseline in South Asians was measured to be 847 pg/ml on day 0 (Figure 5.8). 1-AG level was decreased to 726 pg/ml, 508 pg/ml, 651 pg/ml, 618 pg/ml, 713 pg/ml and 467 pg/ml on days 4, 7, 14, 28, 35 and 39 respectively (Figure 5.8, compare day 0 to days 4, 7, 14, 28, 35 and 39). On the contrary, 1-AG level was increased to 895 pg/ml on day 21 compared to its baseline level on day 0 (Figure 5.8, compare day 0 to day 21). Overall, there were no significant changes in 1-AG level between all the time points of UVR exposure in the data obtained from both groups this experiment.

**Figure 5.8.** 1-AG levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.9. Effect of UVR on docosapentaenoylethanolamide

Docosapentaenoylethanolamide (DPEA) level in White Caucasians was measured to be 76pg/ml (day 0, Figure 5.9). Overall, DPEA levels were similar among all the time points of UVR exposure compared to the baseline on day 0. (Figure 5.9, compare day 0 to day 4, 7, 14, 21, 28, 35 and 39). DPEA baseline level in South Asians was assessed to be 48 pg/ml (day 0, Figure 5.9). Also, there were no important changes in DPEA levels, they were similar between all the time point of UVR exposures compared to the baseline on day 0. (Figure 5.9, compare day 0 to day 4, 7, 14, 21, 28, 35 and 39). Hence, no significant differences in DPEA levels were found in this experiment according to One-Way ANOVA test used for both groups.

![DPEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.](image)

**Figure 5.9.** DPEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.10. Effect of UVR on serum Myristylethanolamide

Myristylethanolamide (MEA) level in White Caucasians was considered to be 465pg/ml on day 0, whereas on day 4, 7 and day 28, MEA levels were increased to 723pg/ml, 599pg/ml and 793pg/ml respectively (Figure 5.10, compare day 0 to day 4, 7 and day 28). After that, MEA levels were decreased on day 14, 21, 35 and 39 to 374pg/ml, 541pg/ml, 449pg/ml and 403pg/ml respectively, compared to the baseline level (Figure 5.10, compare day 0 to day 14, 21, 35 and 39). MEA baseline level in South Asians was considered to be 335pg/ml on day 0. Compared to the baseline, MEA levels were increased to 337pg/ml, 449pg/ml, 773pg/ml, 477pg/ml and 492pg/ml on days 4, 7, 14, 28 and 35 respectively (Figure 5.10, compare day 0 to days 4, 7, 14, 28 and 35). The highest level of MEA was recorded at day 15 (773pg/ml). However, MEA levels were decreased only on day 21 and 39 to 299 pg/ml and 297 pg/ml respectively, compared to the baseline level (Figure 5.10, compare day 0 to day 21 and day 39). Yet, One-Way ANOVA did not show any significant differences in MEA levels between all the time points of UVR exposure in both groups examined. Therefore, this result conclude that exposure to UVR for 6 weeks did not elicit significant changes in MEA level compared to the MEA baseline.

**Figure 5.10.** MEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.11. Effect of UVR on serum dihomo gamma linoleoyalethanolamide

Dihomo gamma linoleoyalethanolamide (DGLEA) level in White Caucasians was assessed to be 25 pg/ml (day 0, Figure 5.11). In general, DGLEA levels were similar between all the time points of UVR exposure compared to the baseline on day 0. (Figure 5.11, compare day 0 to day 4, 7, 14, 21, 28, 35 and 39). DGLEA baseline level in South Asians was calculated to be 20pg/ml on day 0. Compared to the baseline, there were no major changes in DGLEA levels between all the time points of UVR exposure (Figure 5.11, compare day 0 to day 4, 7, 14, 21, 28, 35 and 39). Consequently, no significant differences were seen in DGLEA levels in this experiment. One-Way ANOVA followed by Tukey did not show any significant differences in the DGLEA levels between different time points of UVR exposure in all groups.

Figure 5.11. DGLEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.
5.4.1.12. Effect of UVR on serum eicosapentaenoylethanolamide

Eicosapentaenoylethanolamide (EPEA) was found in 6 out of 10 White Caucasians of skin phototype II. EPEA baseline level was calculated to be 44 pg/ml. This level did not change after the first dose of UVR on day 4 (43 pg/ml). Compared to the baseline on day 0, EPEA levels however increased to 69 pg/ml, 72 pg/ml, 70 pg/ml, 88 pg/ml and 79 pg/ml from the second dose of UVR on day 7 until day 35 respectively (Figure 5.12, compare day 0 to days 7, 14, 21, 28 and 35). On day 39, EPEA level decreased to the baseline level (45 pg/ml). These data were analyzed using One-Way ANOVA to investigate the differences in the EPEA levels between different time points of UVR exposure. Statistically, there were no significant differences in EPEA levels after 6 weeks of UVR exposure, compared to the EPEA baseline.

![Figure 5.12. EPEA levels in serum before and after multiple UVR exposures (1.35 standard erythemal dose) in White Caucasians (skin phototype II, n=10 volunteers) and South Asians (skin phototype V, n = 6 volunteers). Data shown as mean ± SD of n = 3 experiments. Comparing data to control.](image)
5.5. Discussion

The aim of this study was to determine the effect of UVR on human serum endocannabinoids and NAE using volunteers with two different skin phototypes. We wanted to see if there are any differences in serum endocannabinoids and NAE in response to multiple doses of UVR. The study was carried out for 6 weeks to mimic UVR exposure during summer time. All clinical work was undertaken by Prof Rhodes’ group.

Based on our data, there were differences in the baseline levels of the endocannabinoids and NAEs in the White Caucasians and South Asians. However, findings from this study showed that UVR had no effect on PEA, LEA, STEA and DGLEA levels in either skin phototypes. Regardless of the differences in the baseline between the two tested skin types, multiple doses of UVR approximately induced similar fluctuating effects on PEA, LEA, STEA and DGLEA levels in both skin types.

In general, NEA levels were found at higher concentrations in White Caucasians of skin phototype II than South Asians of phototype V. PEA for example was at the highest level among the detected NAE then DHEA, OEA, LEA, STEA, MEA, AEA, DP-EA, and DGLEA. Moreover, DGLEA was at the lowest level in all the NAEs found. Its levels were under 30 pg/ml in both groups. On the other hand, EPEA was found in 6 out of 10 volunteers of the White Caucasian group while, it was not found in the South Asian group. UVR increased 2-AG in the serum of both groups. However, 2-AG levels were higher in the South Asian group compared to the White Caucasian group. In all the time points including the day 0 (baseline), no significant differences were recorded between those time points in both examined groups. Therefore, based on these observations it was concluded that UV radiation at the multiple doses used in this clinical study has no effect on the serum endocannabinoids and their congeners in both skin phototype used. Therefore, further study is still needed.

Sample stability could be another issue behind the negative results showed here in our study. The human serum samples for the clinical study were stored at -80°C. Although, all endocannabinoids and NAEs were detected by LC-MS/MS analysis, the samples
may be affected by storage. Especially, with NAEs which have very low levels in intact cells and tissue (Berdyshev et al., 2000). Furthermore, it is well known that the endocannabinoids and NEA are usually produced on demand rather than being stored in cells (Schreiber et al., 2007). Also, the levels of such of these NAE are significantly reduced with samples freezing and thawing (Schreiber et al., 2007). Although this was not an issue in this study, since the samples were only defrosted once, for the analysis. Apart from this, endocannabinoid and NAE levels were higher in skin phototype II than skin phototype V.

As it was mentioned before, the baseline of these endocannabinoids and NAE were different thus, the differences in their baseline levels may attributed to other factors such as the dietary habits of the participants but not to the UVR itself. It is well known that diet has a large effect on endocannabinoid and NAE levels in our body. Since the examined groups in this study were from different ethnic groups, they could have different life styles especially in the way of food consumption.

The current study showed that the levels of OEA, DHEA, AEA and DPEA were higher in the White Caucasians of skin phototype II than the South Asians of phototype V. This result may be due to the diet of the White Caucasians volunteers, especially food that contains omega-3 PUFA such as DHA and EPA. Dietary fatty acids can exert multiple, indirect influences on lipid metabolism across n-3, n-6, n-9 pathways through processes including tissue fatty acid uptake, distribution, and oxidation, fatty acid circulation and elimination rates (Rapoport, 2008, Rapoport et al., 2007, Madsen et al., 1999, Polozova et al., 2006, Emken et al., 1993). Further support for this suggestion comes from the observation that DHA has been reported to induce an increase in plasma concentrations of its NAE derivative DHEA (Wood et al., 2010). In addition, another study, reported that two week supplementation with DHA affected brain and plasma NAE and endocannabinoid metabolites to favour the formation of DHA and EPA derivatives. Specifically, DHEA and EPG concentrations increased in both plasma and brain, whereas AEA decreased in brain and OEA, AG, OG and AA levels decreased in plasma (Wood et al., 2010). All these data suggest that competition among lipid biosynthetic enzymes utilizing fatty acids as substrates could represent an important influence on
the formation of downstream lipid metabolites, including their derived endocannabinoid metabolome constituents (Wood et al., 2010).
CHAPTER 6: General discussion and future studies

6.1. Introduction

The skin is the largest organ of the human body. It forms the physical barrier that protect the body from environmental stress and regulates the inward and outward passage of water (Hunter, 2003). Skin has also a protective role against microorganisms, chemical agents and UVR. It is well known that UV radiation is a dangerous environmental stressor on the skin. Repeated UVR exposure especially due to higher levels of UVR reaching the earth, makes the skin impairs its protective function and as a consequence, skin inflammation, DNA damaging and skin cancer may develop. Therefore, finding an alternative way to protect our skin from harmful UV radiation is important. Based on several investigations dietary n-3 PUFA could be a reliable way offering protection against an array of health disorders. N-3 PUFA are now some of the most studied fatty acids that have been thought to play a key role as anti-inflammatory agent. N-3 PUFA can regulate a wide range of functions in the body, including blood pressure, blood clotting, and correct development and functioning of the brain and nervous systems (Das, 2006). Furthermore, they have a role in regulating inflammatory responses through the production of inflammatory mediators such as the eicosanoids (Das, 2006, Calder, 2006). EPA and DHA are believed to have several health benefits (Wall et al., 2010). There is evidence that n-3 PUFAs improve cardiovascular outcomes (De Caterina, 2011) and that they have efficacy in rheumatoid conditions (Bhangle and Kolasinski, 2011). There is now emerging evidence that n-3 PUFAs may also have anti-cancer activities (Cockbain et al., 2012). All these discoveries suggest that n-3 PUFA could play a role in protecting the human body from the harmful effects of environmental stressors like UV radiation.

N-3 PUFA can also generate endocannabinoids which are a group of lipid mediators, derived from fatty acid precursors linked to an ethanolamine moiety. They can also stimulate various physiological effects including hypothermia, anti-nociception, vasodilation and anti-inflammatory effects (Di Marzo, 1998, Zygmunt et al., 1999) and have been implicated in several physiological functions and conditions ranging from analgesia, reproduction, modulation of vascular tone, obesity, cancer, schizophrenia,
and multiple sclerosis (Pacher et al., 2006). Endocannabinoid congeners such as the NAEs include palmitoylethanolamide (PEA), oleoylthanolamide (OEA) and stearoylthanolamide (STEA). There are also involved in several physiological and pathophysiological processes, such as, inflammation, pain, sleep, reproduction and drug addiction (Piomelli, 2003, Lo Verme et al., 2005, Fu et al., 2003). Therefore, our study was set out to explore the effect of n-3 PUFA on endocannabinoids and their metabolizing enzymes in human skin cells exposed to UV radiation. The study has also sought to know whether the n-3 PUFA and UVR could have an effect on the formation of endocannabinoids and their related fatty acids. In order to address these questions, this study was carried out on two types of human skin cells lines, HaCaT keratinocytes and 46BR.IN fibroblasts. Furthermore, a clinical study has taken place to investigate the effect of UV radiation on serum endocannabinoids and other NAE.

6.2. General discussion
Generally, our results show that the DHA treatment increased the intracellular levels of endocannabinoid and NAEs such as AEA, PEA and DHEA in HaCaT keratinocytes. Whereas, in 46BR.IN fibroblasts DHA was more effective and increased all the detected endocannabinoid and their congeners including STEA, PEA, OEA, DHEA, AEA and 2-AG. These results are in the same line with other studies which demonstrated that DHA supplementation increased AEA and DHEA levels (Berger et al., 2001, Artmann et al., 2008). On the other hand, EPA treatment decreased OEA, PEA, LEA and DHEA in HaCaT keratinocytes and 46BR.IN fibroblasts. Whereas, the intracellular levels of AEA and 2-AG did not change in both cells model but 2-AG level may increase in 46BR.IN fibroblasts. These results are also supported with the observations in Artmann study showed that EPA and DHA decreased the intracellular levels of the 2-AG, PEA, STEA and OEA but not AEA and DHEA (Artmann et al., 2008). Furthermore, AEA and 2-AG also have been reported to be decreased in 3T3-F442A adipocytes after DHA treatment (Matias et al., 2008).
UV radiation was found to decrease the intracellular of STEA, PEA and LEA in both HaCaT keratinocytes and 46BR.IN fibroblasts in the presence and absence of n-3 PUFA supplementations. However, the intracellular levels of DHEA, AEA and 2-AG were observed to fluctuate in both cells lines post UV radiation. The extracellular levels of OEA and 2-AG were found to be increased whereas, STEA and DHEA levels did not change in the irradiated HaCaT keratinocytes and 46BR.IN fibroblasts. All these finding showed that UV radiation may be affect the formation of the endocannabinoids and their related compounds. These findings may attributed to phospholipase A₂ (PLA₂) activity that is induced after UVR exposure (Chen et al., 1996, Cohen and DeLeo, 1993, Hanson and DeLeo, 1990), as PLA₂ has been reported to be one of the possible biosynthetic routes of NAEs via N-acyl-lysoPE (Sun et al., 2004). Moreover, UVR has been reported to stimulate N-acyl-PE and NAEs including AEA (Berdyshev et al., 2000).

Data from a second study showed that UVR-induced COX-2 up-regulation. In fact, this observation is well documented, for example, several studies suggested the up-regulation of COX-2 protein levels mRNA and following UVB irradiation in HaCaT keratinocytes, in human keratinocytes in volunteers subjected to UVB radiation, in mouse epidermis and in the skin of hairless mice (Buckman et al., 1998, Tang et al., 2001, Bachelor et al., 2005, Kim et al., 2007).

Our results also showed that DHA treatment significantly decreased COX-2 expression in 46BR.IN fibroblasts in dose dependent manner. UVR-induced COX-2 expression was also dose dependently inhibited by DHA treatment in 46BR.IN fibroblasts. Similarly, but in different cells, DHA was reported to block UVR induced COX-2 expression (Sengupta et al., 2003, Zhang and Bowden, 2008, Rahman et al., 2011). In contrast, DHA did not inhibit UVR-induced COX-2 expression in HaCaT keratinocytes in our study. These results are also in agreement with another study used different stimulus and different type of cells. They found that DHA enhanced phorbol ester or interleukin 1β-induced COX-2 expression in rat vascular endothelial cells (Machida et al., 2005).

Data from our study showed that EPA decreased COX-2 protein levels in a concentration dependent manner in both HaCaT keratinocytes and 46BR.IN fibroblasts
cells. These results are in agreement with another studies where EPA induced down-regulation in COX-2 expression (Calviello et al., 2004, Kim et al., 2006). In addition, our results suggest that EPA did not prevent UVR-induced COX-2 expression in either cell line.

Back to endocannabinoid, our results revealed that UVR induced down-regulation in FAAH levels while, there were no consistent effects on NAPE-PLD in HaCaT keratinocytes and 46BR.IN fibroblasts. However, AEA and 2-AG synthesis was found to be increased after UVR exposure in both cell lines used in this study.

DHA and EPA treatment decreased FAAH level and increased NAPE-PLD levels in both HaCaT keratinocytes and 46BR.IN fibroblasts. This was consistent with our results showing that DHA increased AEA production in HaCaT keratinocytes as observed in another part of this study.

Data from the present study also indicated that UV radiation could inhibit NAPE-PLD expression. These data are in line with another research reported that LPS inhibits NAPE-PLD expression (Zhu et al., 2011). In fact, these observations may be due to many reasons, such as gene expression changes induced due to UV radiation.

In the clinical study, our findings showed that UVR has no significant effects on PEA, LEA, STEA, PEA, LEA and DGLEA levels in either skin phototype (II or V). However, 2-AG levels were found increased in both skin phototypes post UV radiation. In general, NEA levels were higher in the White Caucasian of skin phototype II than the South Asian of phototype V. PEA for example was the most abundant NAE among the detected NAEs while, DGLEA was at the lowest level between all the found NAEs. EPEA was found in 6 out of 10 volunteers of the White Caucasian group. 2-AG level was higher in the South Asian group while, DHEA levels were much higher in White Caucasians group than South Asians group. All these observations suggest that these differences between two groups of volunteers can be attributed to diet and not UVR because there were huge difference in the baseline levels of endocannabinoid and NAE. Overall, the main findings of this project are:
1. N-3 PUFA increased NAPE-PLD levels in HaCaT keratinocytes and 46BR.IN fibroblasts and as a result increased the production of endocannabinoids and NAE.

2. N-3 PUFA reduced FAAH levels in HaCaT keratinocytes and 46BR.IN fibroblasts which could be an indirect way to increase the concentration of endocannabinoids and NAE.

3. N-3 PUFA induced AEA and 2-AG biosynthesis in HaCaT keratinocytes and 46BR.IN fibroblasts

4. DHA treatment significantly decreased COX-2 expression in 46BR.IN fibroblasts

5. DHA inhibited UVR-induced COX-2 overexpression in 46BR.IN fibroblasts

6. DHA induced COX-2 up-regulation in HaCaT keratinocytes

7. DHA did not prevent UVR induced COX-2 up-regulation in HaCaT keratinocytes.

8. EPA induced COX-2 down-regulation in HaCaT keratinocytes and 46BR.IN fibroblasts.

9. EPA did not prevent UVR induced COX-2 up-regulation in both HaCaT keratinocytes and 46BR.IN fibroblasts.

10. UVR did not have any effect on endocannabinoid and NAE biosynthesis. However, UVR induced endocannabinoids production in some experiments and this could suggest a defense mechanism of skin cells followed UV radiation.

11. UVR had no major effect on human serum NAE in both skin phototypes II and V

12. UVR increased 2-AG in human serum NAE in both skin phototypes II and V

13. Human serum NAE levels were found to be higher in White Caucasian group (skin phototypes II).

14. Human serum 2-AG level was higher in the South Asian group (skin phototypes V).
6.3. Future directions

This study has shown that n-3 PUFA increased endocannabinoids and NAE in human skin cells. Also our results showed that n-3 PUFA increased NAPE-PLD expression and decreased FAAH in the human cell lines HaCaT keratinocytes and 46BR.IN fibroblasts. These results indicated that n-3 PUFA may alter endocannabinoid biosynthesis pathways in two ways; (a) directly by increasing NAPE-PLD which in turn increases the biosynthesis of endocannabinoids and NAE. (b) Indirectly by decreasing FAAH, as a result increasing the intracellular levels of endocannabinoids. However, it will be of interest to elucidate the effect of these n-3 PUFA on the 2-AG synthesizing enzyme diacylglycerol lipase (DAGL) and the 2-AG degrading enzyme monoacylglycerols lipase (MAGL) in HaCaT keratinocytes and 46BR.IN fibroblasts in the presence and absence of UVR. Furthermore, it will be essential to investigate the effect of n-3 PUFA in NAPE-PLD, FAAH, DAGL and MAGL in primary HaCaT keratinocytes and in 46BR.IN fibroblasts in the presence and absence of UVR. Also, it is importance to assess the effect of UVR on the biosynthesis of endocannabinoids and NAE through measuring Ca\(^{2+}\) intracellular levels per and post UVR.

Data from this study also showed that DHA supplementation significantly decreased COX-2 expression and inhibited UVR-induced COX-2 up-regulation in 46BR.IN fibroblasts. In contrast, in HaCaT keratinocytes, DHA exerts an opposite effect by increasing COX-2 expression and did not prevent UVR-induced COX-2 expression in the irradiated HaCaT keratinocytes. Moreover, our results showed that EPA induced COX-2 down-regulation in HaCaT keratinocytes and 46BR.IN fibroblasts. However, EPA did not inhibit UVR-induced COX-2 expression in the HaCaT keratinocytes and 46BR.IN fibroblasts. Therefore, further study is still required to assess the effect of DHA and EPA on COX-2 expression in the presence and absence of UVR in primary HaCaT keratinocytes and in 46BR.IN fibroblasts.

Our study did not produce results that could help us to assess the effect of n-3 PUFA on 12- and 15-LOX in HaCaT keratinocytes and 46BR.IN fibroblasts. Therefore, more work is needed to determine the effect of n-3 PUFA on 12- and 15-LOX in these cells and in the presence and absence of UVR. Furthermore, it will be interesting to clarify
the effect of exogenous AEA and 2-AG on the expression of COX-2, 12-LOX and 15-LOX in HaCaT keratinocytes and 46BR.IN fibroblasts in the presence and absence of UVR.

Several studies reported that n-3 PUFA and UVR have an effect on gene expression on different cells and tissues. Further work aiming to examine NAPE-PLD, FAAH, DAGL and MAGL gene expression in HaCaT keratinocytes and 46BR.IN fibroblasts, in the presence and absence of n-3 PUFA and UVR is needed.

Finally, our results showed that UVR has no major effects on circulating levels of endocannabinoids and NAE as measured in human serum samples. We believed that the differences observed in endocannabinoids and NAE may be attributed to dietary habits of the volunteers engaged in the clinical study. Therefore, it will be essential to assess the effect diet on endocannabinoid and NAE in the human circulation.
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Appendices

Appendix 1

1. Fatty acid stock solution

1.1. Oleic Acid (OA) stock solution (50mM)
   1. OA 25 mg
   2. Dimethyl sulfoxide (DMSO) 1.8 ml
   3. Aliquot to 50µl each and store at -20°C.

1M of OA = 282.46 g, so 14.123g are needed to prepare OA at concentration of 50mM.

1.2. Eicosapentaenoic Acid (EPA) stock solution (50mM)
   1. EPA 25 mg
   2. Dimethyl sulfoxide (DMSO) 1.7 ml
   3. Aliquot to 50µl each and store at -20°C.

1M of EPA = 302.45 g, so 15.123g are needed to prepare EPA at concentration of 50mM

1.3. Docosahexaenoic Acid (DHA) stock solution (50mM)
   1. Docosahexaenoic acid 25 mg
   2. Dimethyl sulfoxide (DMSO) 1.5 ml
   3. Aliquot to 50µl each and store at -20°C.

1M of DHA = 328.49 g, so 16.424g are needed to prepare DHA at concentration of 50mM.

Appendix 2

2.1. Preparation of BSA standards

Prepare BSA solutions in 100 µl

1. The stock solution of BSA is 1.54 mg/ml
2. The required concentrations are 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml
Example: \((0.1 \text{mg/ml}) / (1.54 \text{ mg/ml}) = 0.06 \times 100\mu l = 6 \mu l\)

6 \mu l BSA + 94 \mu l of sample buffer (Appendix 3, section 3.1.5) = 100\mu l

3. Do this calculation of the rest of required concentrations
4. Put in eppendroff tubes and vortex
5. Store in -20 °C to be used for the protein assay

2.2. Genesis software (Version 2)

1. In a 96-well microplate add 5 \mu l of each BSA standard and 5 \mu l of each test sample all in triplicate
2. Add 25 \mu l Protein Assay Reagent A (Bio-Rad) in each well
3. Add 200 \mu l Protein Assay Reagent B (Bio-Rad) in each well
4. Leave the plate to stand in the dark for 15 min
5. Read the absorbance of all wells using a plate reader at 650 nm
6. To estimate the protein content, launch Genesis software (Version 2).
7. Once opened, click on ‘Protocol’ at the menu bar and then ‘Open’.
8. From the list, choose ‘protein.prt’ and confirm the selection ‘OK’.
9. Once set up, gently place the micro-plate onto the reader holder and run samples (running man icon at the task bar).
10. Type any relevant comments and confirm by ‘RUN’. Note: Filter must be set at 650 nm.
11. Save data.

2.3. Sample calculation for adjusting the protein content of the cell lysate

Example: if the sample volume is 200 \mu l and the protein concentration of the sample is 3.7 \mu g/\mu l, then a dilution to a concentration of 2 \mu g/\mu l is needed.

So: \(C_1V_1 = C_2V_2\)

\(V_2 = (3.7 \mu g/\mu l \times 200 \mu l) / (2 \mu g/\mu l) = 370 \mu l\)

370 \mu l - 200 \mu l = 170 \mu l.
Therefore; 170 µl of sample buffer (Appendix 3, section 3.1.5) should be added to the original lysate to generate a sample of: volume 370 µl, protein concentration 2µg/µl.

Appendix 3

3.1 Preparation of buffers and stains

3.1.1. Tank (running) buffer: (PH 8.4-8.5) 1 litre

1. Tris base 30 g
2. Glycine 144 g
3. SDS 10 g
4. Add dH$_2$O to adjust the volume to 1 litre

Do not adjust PH

In case of preparing 1 L, mix all solids together, add dH$_2$O up 1000 ml using a stirrer mix very gently to dissolve all ingredients.

3.1.2. Transblotting buffer: (PH 8.4-8.5) 1 litre

1. Tris base 30 g
2. Glycine 144 g
3. dH$_2$O to adjust the volume to 1 litre. Do not adjust PH

In case of preparing 1 L, mix all solids together, add dH$_2$O up 1000 ml using a stirrer mix very gently to dissolve all ingredients.

3.1.3. Lower (separating) gel Tris buffer (PH 8.8) - 0.5 litre

1. Tris base 90.8 g
2. SDS 2 g
3. dH$_2$O 500 ml

Adjust PH using concentrated HCl
3.1.4. Upper (stacking) gel Tris buffer (PH 6.9) - 0.5 litre

1. Tris base 30.3 g
2. SDS 2 g
3. dH$_2$O 500 ml

Adjust pH using concentrated HCl (Add few drops until pH adjusted)

3.1.5. Sample buffer 25 ml

10% Glycerol, 2.5 ml

0.02M EDTA, 0.185 g

MW = 372.24 g

1M = 372.24 g/L

Quantity required is 0.02 M in 100 ml

\[
(0.02M \times 372.24 \text{ g}) / 1M = 7.44 \text{ g}
\]

\[
(7.44 \text{ g} \times 100 \text{ ml}) / (1000 \text{ ml}) = 0.74 \text{ g}
\]

To dissolve EDTA add few drops of NaOH 1M. 1M = 40 g/1000ml

(1mM = 4 g/100 ml)

6% SDS, 1.5g

62.4mM Tris base, 0.19g

MW = 121.14 g

1M = 121.14 g/L

Quantity required is 62 mM in 100 ml

1M = 121.14 g

\[
(0.062M \times 121.14 \text{ g}) / 1M = 7.51 \text{ g}
\]

\[
(7.51 \times 100 \text{ ml}) / (1000 \text{ ml}) = 0.76 \text{ g}
\]
Bromophenol blue 0.08% (omit this step and do it when adding samples)

dH₂O to adjust the volume to 25ml

3.1.6. Coomassie blue stain for SDS

1. 125 ml dH₂O
2. 100 ml methanol
3. 25 ml acetic acid
4. 0.1% w/v coomassie blue

Filter before use with Whatman filter paper and funnel.

3.1.7. Destain for SDS

1. 250 ml dH₂O
2. 200 ml methanol
3. 50 ml glacial acetic acid

3.1.8. Fast green 0.1% for PVDF membrane

1. 0.5 g fast green
2. 500 ml dH₂O Filter with Whatman filter paper and funnel

3.1.9. ECL solution

1. Tris base 2.5 ml (pH 8.5)
2. Luminal 130 µl
3. P-coumaric acid 60 µl
4. H₂O₂ 28 µl
5. dH₂O 22.32 ml.

The mixture was protected from the light by mixed in 50 ml universal tube wrapped in foil.
Appendix 4

4.1. Membrane stripping

4.1.1. Mild stripping buffer

1. Glycine 1.5 g
2. SDS 0.1 g
3. Tween 20 (1ml)

Adjust pH to 2.2 by using concentrated HCl (Add few drops until pH adjusted) Bring volume up to 100 ml with dH2O.

4.2. Protocol of stripping western blots for re-probing

1. Put the PVDF membrane in a 10cm square petri dish contains of an enough volume of mild stripping buffer that will cover the PVDF membrane, usually use 15 – 20 ml.
2. Incubate at room temperature for 10 min.
3. Discharge mild stripping buffer.
4. Incubate PVDF membrane again at room temperature for 10 minutes with fresh stripping buffer.
5. Discharge buffer.
6. Incubate PVDF membrane at room temperature for 10 min with PBS
7. Pour off the PBS
8. Incubate PVDF membrane again at room temperature for 10 min with PBS
9. Pour off the PBS
10. Incubate PVDF membrane at room temperature for 5 min with PBS/Tween20 (0.05%)
11. Pour off the PBS/Tween20 (0.05%)
12. Incubate PVDF membrane again at room temperature for 5 min with PBS/Tween20 (0.05%)
13. Pour off the PBS/Tween20 (0.05%)
14. Incubate PVDF membrane at room temperature for 5 min with PBS
15. Ready for blocking stage.

Appendix 5

5.1 Chloroform/methanol (2:1 ratio)
1. Add 100 ml methanol (HPLC grade) into 500 measuring flask.
2. Add 300 ml of chloroform (HPLC grade) to step number 1
3. Store at room temperature for use, this solution can stand up to one month

5.2 Internal standards (1 ng/µl AEA-d8 and 1 ng/µl 2-AG-d8)
1. In an amber vial, mix 100 µl of 100 ng/µl stocks with 900 µl ethanol (HPLC grade)
   to make a 10 ng/µl stock
2. In another amber vial, mix 100 µl 10 ng/µl stocks and mix with 900 µl ethanol
   (HPLC grade) to make a 1 ng/µl stock
3. Seal both 1 ng/µl stock and remaining 10 ng/µl stocks with Parafilm and store at -
   20 ºC for up to three months

5.3 Endocannabinoid cocktail for calibration line (400 pg/µl)
1. Using a Hamilton syringe, measure 40 µl of the 10 ng/µl AEA standard into an
   amber vial
2. Add 40 µl each of the 10 ng/µl 2-AG, ALEA, DHEA, OEA, SEA, PEA, EPEA
   and LEA standards, rinsing the Hamilton syringe between standards
3. Add 640 µl ethanol (HPLC grade) to make up to 1 ml
4. Mix thoroughly
5.4. Mobile Phase A for endocannabinoid analysis

1. Measure 20 ml acetonitrile (HPLC grade) into a 1 L measuring cylinder
2. Add 5 ml glacial acetic acid (HPLC grade)
3. Make up to 1 L with deionized water
4. Vacuum filter
5. Transfer to 1 L bottle

Should be made fresh at the beginning of each run

5.5. Mobile Phase B for endocannabinoid analysis

1. Measure 20 ml deionized water into a 1 L measuring cylinder
2. Add 5 ml glacial acetic acid (HPLC grade)
3. Make up to 1 L with acetonitrile (HPLC grade)
4. Vacuum filter
5. Transfer to 1 L bottle

Should be made fresh at the beginning of each run

5.6. Seal wash

1. Measure 100 ml acetonitrile (HPLC grade) into a 1 L measuring cylinder
2. Make up to 1 L with deionized water
3. Vacuum filter
4. Transfer to 1 L bottle
Should be made fresh at the beginning of each run

5.7. Needle Wash
1. Measure 300 ml deionized water into a 1 L measuring cylinder
2. Make up to 1 L with acetonitrile (HPLC grade)
3. Vacuum filter
4. Transfer to 1 L bottle

Should be made fresh at the beginning of each run

5.8. Shutdown solution I
1. Measure 500 ml methanol (HPLC grade) into a 1 L measuring cylinder
2. Add 2 ml glacial acetic acid (HPLC grade)
3. Make up to 1 L with deionized water
4. Vacuum filter
5. Transfer to 1 L bottle

Should be made fresh at the beginning of each run

5.9. Shutdown solution II
1. Measure 500 ml methanol (HPLC grade) into a 1 L measuring cylinder
2. Make up to 1 L with deionized water
3. Vacuum filter
4. Transfer to 1 L bottle
Should be made fresh at the beginning of each run

Appendix 6

Table 6.1. Comparison between PEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
<thead>
<tr>
<th>Days of UVR exposure</th>
<th>Skin phototype II</th>
<th>Skin phototype V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>3160</td>
<td>2814</td>
</tr>
<tr>
<td>Day 4</td>
<td>4048</td>
<td>2571</td>
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<td>Day 7</td>
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<td>Day 14</td>
<td>3049</td>
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<td>Day 21</td>
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<td>3184</td>
<td>2871</td>
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<td>Day 39</td>
<td>3251</td>
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Table 6.2. Comparison between LEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
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<th>Skin phototype V</th>
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<td>Day 4</td>
<td>896</td>
<td>916</td>
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<td>Day 14</td>
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<td>Day 28</td>
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<td>Day 39</td>
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Table 6.3. Comparison between OEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
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<td>Day 39</td>
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Table 6.4. Comparison between STEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

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<td>Day 39</td>
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Table 6.5. Comparison between DHEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
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<tr>
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<td>2605</td>
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Table 6.6. Comparison between AEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

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<th>Days of UVR exposure</th>
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Table 6.7. Comparison between 2-AG levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

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<th>Days of UVR exposure</th>
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<th>Skin phototype V 2-AG (pg/ml)</th>
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<td>Day 35</td>
<td>1527</td>
<td>1682</td>
</tr>
<tr>
<td>Day 39</td>
<td>1231</td>
<td>1185</td>
</tr>
</tbody>
</table>

Table 6.8. Comparison between 1-AG levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
<thead>
<tr>
<th>Days of UVR exposure</th>
<th>Skin phototype II 1-AG (pg/ml)</th>
<th>Skin phototype V 1-AG (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>742</td>
<td>847</td>
</tr>
<tr>
<td>Day 4</td>
<td>771</td>
<td>726</td>
</tr>
<tr>
<td>Day 7</td>
<td>765</td>
<td>508</td>
</tr>
<tr>
<td>Day 14</td>
<td>690</td>
<td>651</td>
</tr>
<tr>
<td>Day 21</td>
<td>564</td>
<td>895</td>
</tr>
<tr>
<td>Day 28</td>
<td>710</td>
<td>618</td>
</tr>
<tr>
<td>Day 35</td>
<td>684</td>
<td>713</td>
</tr>
<tr>
<td>Day 39</td>
<td>865</td>
<td>467</td>
</tr>
</tbody>
</table>
Table 6.9. Comparison between DP-EA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
<thead>
<tr>
<th>Days of UVR exposure</th>
<th>Skin phototype II</th>
<th>Skin phototype V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>76</td>
<td>43</td>
</tr>
<tr>
<td>Day 4</td>
<td>67</td>
<td>48</td>
</tr>
<tr>
<td>Day 7</td>
<td>73</td>
<td>47</td>
</tr>
<tr>
<td>Day 14</td>
<td>73</td>
<td>53</td>
</tr>
<tr>
<td>Day 21</td>
<td>71</td>
<td>43</td>
</tr>
<tr>
<td>Day 28</td>
<td>81</td>
<td>66</td>
</tr>
<tr>
<td>Day 35</td>
<td>75</td>
<td>63</td>
</tr>
<tr>
<td>Day 39</td>
<td>73</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 6.10. Comparison between Myristoyl-EA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
<thead>
<tr>
<th>Days of UVR exposure</th>
<th>Skin phototype II</th>
<th>Skin phototype V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>465</td>
<td>335</td>
</tr>
<tr>
<td>Day 4</td>
<td>723</td>
<td>337</td>
</tr>
<tr>
<td>Day 7</td>
<td>599</td>
<td>449</td>
</tr>
<tr>
<td>Day 14</td>
<td>374</td>
<td>773</td>
</tr>
<tr>
<td>Day 21</td>
<td>541</td>
<td>299</td>
</tr>
<tr>
<td>Day 28</td>
<td>793</td>
<td>477</td>
</tr>
<tr>
<td>Day 35</td>
<td>449</td>
<td>492</td>
</tr>
<tr>
<td>Day 39</td>
<td>403</td>
<td>297</td>
</tr>
</tbody>
</table>

Table 6.11. Comparison between DGLEA levels in White Caucasians of skin phototype II and South Asians of phototype V pre and post UVR.

<table>
<thead>
<tr>
<th>Days of UVR exposure</th>
<th>Skin phototype II</th>
<th>Skin phototype V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Day 4</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Day 7</td>
<td>26</td>
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<tr>
<td>Day 14</td>
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<td>26</td>
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<tr>
<td>Day 21</td>
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<tr>
<td>Day 28</td>
<td>26</td>
<td>25</td>
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<tr>
<td>Day 35</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>Day 39</td>
<td>22</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 6.12. Comparison of different time point of UVR exposure on EPEA levels in White Caucasians of skin phototype II.

<table>
<thead>
<tr>
<th>Days of UVR exposure</th>
<th>EPEA (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>43</td>
</tr>
<tr>
<td>7</td>
<td>69</td>
</tr>
<tr>
<td>14</td>
<td>72</td>
</tr>
<tr>
<td>21</td>
<td>70</td>
</tr>
<tr>
<td>28</td>
<td>88</td>
</tr>
<tr>
<td>35</td>
<td>79</td>
</tr>
<tr>
<td>39</td>
<td>45</td>
</tr>
</tbody>
</table>