

Efeito da Radiação Ultravioleta B no Sistema Endocanabinoide Cutâneo
Effect of Ultraviolet B Radiation on Cutaneous Endocannabinoid System

Sofia Beatriz Loureiro Marques de Vasconcelos Magina Silva Ramos

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List of abbreviations

ACEA arachidonoyl-2-chloroethylamide

AEA anandamide

2-AG 2-arachidonoylglycerol

BMI body mass index

Ca²⁺ calcium

CB₁ cannabinoid receptor 1

CB₂ cannabinoid receptor 2

COMT catechol-*O*-methyltransferase

COX-2 cyclooxygenase-2

CPDs cyclobutyl pyrimidine dimers

CRP C-reactive protein

hsCRP high sensitivity C-reactive protein

DAG diacylglycerol

DGL diacylglycerol lipase

FAAH fatty acid amide hydrolase

IL interleukin

IP3 inositol-1,4,5-phosphate

MAGL monoacylglycerol lipase

MAPK mitogen-activated protein kinase

MC₁ melanocortin-1 receptor

MITF microphthalmia-associated transcription factor

α-MSH α-melanocyte-stimulating hormone

NF-κB nuclear factor kappa B

OEA oleylethanolamine

PEA palmitoylethanolamide

PIP₂ phosphatidylinositol bisphosphate

PLC phospholipase C

PLC-β phospholipase C-β

PKA protein kinase A

PKC protein kinase C

PKC-β protein kinase C-β

POMC pro-opiomelanocortin

RE retinyl esters

ROS reactive oxygen species

THC Δ^9 tetrahydrocannabinol

TNF α tumor necrosis factor alpha

TRPV1 transient receptor potential vanilloid receptor 1

UV ultraviolet

UVR ultraviolet radiation

UVA ultraviolet A

PUVA psoralen plus UVA

UVB ultraviolet B

nbUVB narrowband ultraviolet B

UVC ultraviolet C

Introduction and Aims

Ultraviolet B radiation

General considerations

Sunlight and its adjacent spectral regions, ultraviolet (UV) and infrared are basic requirements for life on earth and humankind has always been exposed to their influence. Health benefits to sun exposure were reported in ancient times. Several therapeutic actions of UV radiation (UVR) are now supported by sound scientific evidence. However it became clear that solar UVR is also the major environmental insult to the skin.

The word ultraviolet means “beyond violet” and refers to electromagnetic radiation with a wavelength shorter than visible violet light but longer than X-rays (Maverakis *et al.* 2010). UVR represents approximately 5% of terrestrial solar radiation and is divided according to the wavelength into UVA (315-380 nm) separated into UVA₁ (340-380 nm) and UVA₂ (315-340 nm), UVB (280 to 315 nm) and UVC (100 to 280 nm). Solar UVR at sea level is approximately 95-98% UVA and 2 to 5% UVB while UVC is completely absorbed by stratospheric ozone that also attenuates UVB. Little UVB radiation reaches the earth's surface. However, UVB radiation has much more energy than UVA (energy is inversely proportional to wavelength) and is approximately 1000 times more erythemogenic than UVA. In other words, although more UVA reaches the earth, it is mainly UVB that “burns” the skin.

UVR ionizes molecules and induces chemical reactions. When UVR strikes the skin, part is remitted (reflected and scattered), part is absorbed in various layers by molecules termed chromophores, and part is transmitted inward until the energy has been dissipated. Photochemical reactions convert chromophores (proteins, DNA and other components of epidermal cells) into new molecules and these photoproducts activate cellular signal transduction pathways leading to biochemical changes and different cellular responses.

The two major processes limiting the penetration of UVR into skin (absorption and scattering) vary with wavelength (Anderson and Parrish 1981). The depth of penetration is directly proportional to the wavelength of the radiation and UVB is mainly absorbed in the epidermis while UVA can penetrate deep into the dermis (Anderson and Parrish 1981).

An action spectrum indicates which wavelengths produce a photochemical response most effectively. The rate at which the radiant energy is delivered to the skin is expressed as W/cm^2 and is called irradiance. The total radiant energy delivered *per unit* area of skin surface is called the exposure dose or fluence and is the product of irradiance and time:

$$[\text{irradiance (W/cm}^2) \times \text{time (seconds)} = \text{exposure dose (J/cm}^2)]$$

The magnitude of the response to UVR is determined by the exposure dose at a particular wavelength.

Exposure of human skin to UVB results in several distinct pathophysiological responses that may be divided into acute and chronic. The most prominent cutaneous acute effects are the sunburn reaction, tanning, immunosuppression, and vitamin D synthesis. Photoaging and skin cancer are the consequences of chronic exposure. UVR, especially UVB, is a major causal factor for all types of skin cancer and UVB signature mutations in p53 tumour suppressor gene are very prevalent in these tumours (Raj *et al.* 2006).

Acute UVB irradiation causes apoptosis and the term “sunburn cell” refers to keratinocytes undergoing apoptosis. This programmed cell-death of UV-damaged skin cells is a definitive cancer-prevention pathway.

After UVB irradiation, two photoproducts are well recognized, the four-membered ring structure called cyclobutyl pyrimidine dimers (CPDs) that lead to DNA damage (Freeman *et al.* 1989) and previtamin D₃ (Holick *et al.* 1987). The action spectrum for sunburn (erythema) closely correlates with the absorption spectrum of DNA and is within the UVB range.

UVB also increases cyclooxygenase-2 (COX-2) expression in cultured human keratinocytes (Buckman *et al.* 1998; Van Dross *et al.* 2007) and COX-2 protein levels become elevated in the epidermis of human skin following UVB irradiation (Buckman *et al.* 1998).

UVR has been shown to activate phospholipase A₂ in human skin and keratinocytes (De Leo *et al.* 1984), thereby releasing arachidonic acid. UVR also stimulates a biphasic rise in diacylglycerol (DAG) production in melanocytes and keratinocytes resulting from diacylglycerol kinase and phospholipases C and D activation and their action on plasma membrane phospholipids (Punnonen and Yuspa 1992; Carsberg *et al.* 1995).

It is recognized that UVB and UVA radiation enhance reactive oxygen species (ROS) production. This oxidative stress in epidermal cells plays an important role in the photodamage pathway (Portugal-Cohen *et al.* 2011).

UVB increases intracellular calcium (Ca²⁺) level in both keratinocytes cell culture (HaCat cells) and in human skin *in vivo* (Lee *et al.* 2009; Masaki *et al.* 2009). It has been difficult to identify the specific mechanism for UVB-induced Ca²⁺-channel activation. Recently it was demonstrated in keratinocytes (HaCat cells) that UVB radiation increases the intracellular Ca²⁺ via transient receptor potential vanilloid type I (TRPV1) activation (Lee *et al.* 2009). In addition UVB increases TRPV1 expression in both keratinocytes and human skin *in vivo* (Lee *et al.* 2009). A protein kinase C (PKC)-dependent activation of TRPV1 and subsequent Ca²⁺ influx was already suggested (Lee *et al.* 2009). Probably, PKC is not the initial chromophore or target molecule of UVB, but PKC activation occurs by UVB-induced release of DAG, arachidonic acid and ROS (Matsui *et al.* 1996).

UVB activates the nuclear factor kappa B (NF-κB) that is a major inducer of inflammatory response. NF-κB induces the synthesis of proinflammatory interleukins (IL-1, IL-6, IL-8) and tumor necrosis factor alpha (TNFα) by keratinocytes (Wang *et al.* 1995; Portugal-Cohen *et al.* 2011).

Investigating the molecular targets and the signal transduction pathways activated by UVR will increase understanding of both detrimental and therapeutic effects of UVR.

Melanogenesis

Part of this topic has already been reviewed (see Videira I et al 2012, Introduction annex 1)

In the skin, melanin is the first defense against UVR. This pigment is able to absorb and dissipate UVR as harmless heat. The increase of melanin in the epidermis after UV exposure, the so-called tanning, is understood as a host response against future photodamage. Melanogenesis has been the interest of research for many decades, however, until the 1990s the initiating molecular events of tanning were unknown.

UVB and UVA induce tanning by different mechanisms. UVB induces a slow but stable type of pigmentation termed delayed tanning, which requires the increased synthesis of melanin following the stimulation of the entire melanogenic cascade (Alaluf *et al.* 2002; Tadokoro *et al.* 2005). In contrast UVA causes mainly immediate tanning, due to redistribution of melanosomes and generation of ROS via oxidation, polymerization or both of existing melanin or melanin precursors (Maeda and Hatao 2004).

Melanocytes, located in the basal layer of the epidermis, synthesize melanin in discrete organelles, the melanosomes, which once filled with melanin are transferred via long dendritic processes to the surrounding keratinocytes, where they form a supranuclear cap to protect DNA against UVR [reviewed by Schallreuter (Schallreuter 2007)].

The key enzyme in melanogenesis, tyrosinase, is located in melanosomes. The rate of melanin production at baseline and following UVB exposure depends on tyrosinase activity, rather than simply on total tyrosinase protein (Park *et al.* 1999).

Tyrosinase is a copper-containing membrane glycoprotein with the N-terminal located inside the melanosome, whereas the C-terminal sits in the melanocyte cytosol. The enzyme is activated when two serine residues close to C-terminal are phosphorylated by protein kinase C- β (PKC- β). Tyrosinase contains two copper atoms in its active site, and when copper atoms are oxidized the enzyme is inactive. The rate-limiting step in melanogenesis is the oxidation of tyrosine to L-DOPA (Schallreuter 2007). L-DOPA acts as a cofactor and also as a substrate for tyrosinase being oxidized into DOPAquinone. Although the exact interaction between tyrosinase and its substrates is not completely understood, kinetic studies suggest that L-tyrosine and L-DOPA have separate binding sites (Olivares *et al.* 2002; Schallreuter 2007).

Catechol-*O*-methyltransferase (COMT) is present in melanocytes and catalyses the *O*-methylation of catecholic compounds such as L-DOPA (Shibata *et al.* 1993; Smit *et al.* 1994). A potential role of COMT in the regulation of melanogenesis was first suggested in the paper by Axelrod *et al.* (Axelrod and Lerner 1963) and more recently it has been shown that skin tyrosinase activity is significantly increased in hairless COMT-deficient male pups but the role of COMT in melanogenesis is not yet clarified (Forsberg *et al.* 2004).

Melanocytes and keratinocytes interact closely as a structural and functional unit, named “epidermal unit”. Melanogenesis is greater in intact skin or in co-cultures of melanocytes and keratinocytes than in isolated melanocyte cultures, suggesting that keratinocyte-derived products contribute to the UV-induced tanning (Archambault *et al.* 1995; Duval *et al.* 2001). Among the wide variety of autocrine and paracrine melanogenic factors produced by keratinocytes are, melanocyte-stimulating hormone (α -MSH), adrenocorticotrophic hormone (ACTH), endotelin-1, nitric oxide, prostaglandins E_2 and $F_{2\alpha\alpha}$ and granulocyte-macrophage colony stimulating factor (Suzuki *et al.* 1999; Kadekaro *et al.* 2003; Yamaguchi *et al.* 2007; Park *et al.* 2009; Choi *et al.* 2010). These factors can act alone or synergistically with each other to modulate melanocyte function. Some UVR-induced cytokines such as IL-1 and TNF α

inhibit melanogenesis suggesting a fine regulation between melanogenic stimulation and inhibition after UVR (Slominski *et al.* 2004).

The most well known melanogenesis-regulating receptor on melanocytes is the, G-protein-coupled, melanocortin-1 receptor (MC₁). Its activation increases intracellular cAMP and through protein kinase-A (PKA) induces microphthalmia-associated transcription factor (MITF) transcription. MITF in turn upregulates the transcription of tyrosinase, tyrosinase-related-protein-1, tyrosinase-related-protein-2 and PKC- β genes, increasing melanin production (Shibahara *et al.* 2000; Schallreuter 2007; Park *et al.* 2009; Choi *et al.* 2010). α -MSH and ACTH are among the MC₁ agonists.

The key observation of the coinciding spectrum, within the UVB range, for producing a delayed tan (Parrish *et al.* 1982) and for induction of CPDs (Freeman *et al.* 1989) suggested for the first time a cause effect relation between DNA damage and melanogenesis. It was recognized that the tumor-suppressor protein p53 is a transcription factor that plays a pivotal role in stimulating melanogenesis after UVB-induced DNA damage (Khlgatian *et al.* 2002). In keratinocytes, UVB triggers a more than 30-fold increase in the formation of pro-opiomelanocortin (POMC) and of its cleavage product α -MSH (Lin and Fisher 2007; Choi *et al.* 2010). This UVB-induced α -MSH production by keratinocytes is regulated via a p53 consensus sequence in the POMC gene promoter (Cui *et al.* 2007). Keratinocyte-derived α -MSH then stimulates MC₁ on melanocytes, suggesting the important role of keratinocytes for sensing UVR.

Despite the strong influence of MC₁ receptors on skin pigmentation, it is clear that other melanocyte receptors such as β_2 adrenoceptor (Gillbro *et al.* 2004), M₁, M₃ and M₅ muscarinic receptors (Grando *et al.* 2006) and oestrogen receptors (Thornton *et al.* 2006) are also involved in melanogenesis regulation via adenylyclase activation .

UVR induces the release of DAG from plasma membrane lipids, which then activates PKC- β that in turn activates tyrosinase and stimulates melanogenesis (Punnonen and Yuspa 1992; Park *et al.* 1993; Park *et al.* 1999; Schallreuter 2007; Park

et al. 2009; Choi *et al.* 2010). DAG release after UVR may also be mediated indirectly via activation of endothelin-1 receptors or α_1 adrenoceptors in melanocyte cell membranes (Park *et al.* 2009).

Identification of factors that modulate UV-induced melanogenesis could increase opportunities for targeted therapeutics ranging from the development of cosmetics to the prevention of skin cancer.

Therapeutic use

Part of this topic has already been reviewed (see Osório F et al 2012, Introduction annex 2)

UVR has been used in the management of skin diseases with great success and at a constantly increasing rate for decades, since Goeckerman first used it in 1925. Thereby UV phototherapy becomes an essential part of modern dermatological therapy.

There are many types of phototherapy including: broadband UVB (280-320 nm), narrowband UVB (nbUVB) (311-313 nm), UVA₁ (340-400 nm), and combination therapy of UVA (320-400 nm) plus the photosensitizer psoralen (PUVA). More recently handheld phototherapy technologies were developed to deliver UVB targeted to skin lesions.

Psoriasis is the prototypic skin disease with a favourable response to UVB phototherapy. In 1977, Fischer (Fischer 1977) found that ultraviolet light at a wavelength of 313 nm was effective in clearing psoriatic plaques. Few years later, it was determined that the most effective wavelengths were between 295 and 313 nm, indicating that this wavelength may possess the optimal “phototherapy index” for clearing psoriasis (Parrish and Jaenicke 1981). Over the past 30 years, the introduction of fluorescent bulbs with a limited spectrum of 311–313 nm (nbUVB) has marked an advance in psoriasis phototherapy. Its success as therapeutic agent has stimulated studies about the underlying mechanisms involved in resolution of psoriatic plaques.

UVB exerts a multitude of biological effects within the skin, however it remains unclear which of these induce clearance of psoriasis and what is the reason for the marked interpatient variation in clinical responses (Ryan *et al.* 2010). In addition identification of molecular biomarkers of UV sensitivity may facilitate treatment predictability.

The development of psoriasis is dependent on complex interactions between the innate immune system, dendritic cells and activated T-cells driving hyperproliferation of genetically predisposed abnormal keratinocytes. Most of the recent studies have highlighted the nbUVB immunosuppressive effect, leading, in the epidermis, to downregulation of T helper 17 and interferon signaling pathways (Racz *et al.* 2011), reduction of T cells (Krueger *et al.* 1995; Carrascosa *et al.* 2007; Erkin *et al.* 2007), depletion of Langerhans cells (Murphy *et al.* 1993) and induction of T regulatory cells (Schwarz *et al.* 2004). In addition, soluble factors induced upon UVB exposure, such as IL-10, IL-22, IL-17, IL-23, IL-8, vascular endothelial growth factor, TNF α , *cis*-urocanic acid and vitamin D₃, could also be involved in UVB therapeutic effect (Ullrich 1994; Beissert *et al.* 2001; Gorman *et al.* 2007; Cicarma *et al.* 2010; Coimbra *et al.* 2010; Ryan *et al.* 2010). Moreover it was observed that UVB 311nm induces significant keratinocytes apoptosis in lesional epidermis, and keratinocyte apoptosis was proposed as the key mechanism in psoriatic plaques clearance (Aufiero *et al.* 2006; Weatherhead *et al.* 2011).

Patients with moderate to severe psoriasis have been found to be at greater risk of developing comorbidities such as metabolic syndrome, obesity and cardiovascular diseases (McDonald 1989; Wakkee *et al.* 2007; Yiu *et al.* 2011). More recently an effect of phototherapy on inflammatory markers and circulating adipokine levels was described and a relation with clinical benefit was hypothesised (Coimbra *et al.* 2009; Coimbra *et al.* 2010; Kawashima *et al.* 2011; Shibata *et al.* 2011).

Endocannabinoid system

Cannabinoid receptors

In the mid-1960s the main psychoactive component of *Cannabis sativa*, the lipophilic compound Δ^9 tetrahydrocannabinol (THC) was discovered (Mechoulam and Gaoni 1965). More than two decades later, the first THC-specific receptor named cannabinoid receptor 1 (CB₁) was identified in rat brain (Devane *et al.* 1988) and then cloned from mammalian tissues (Matsuda *et al.* 1990). Three years later, in 1993, a second cannabinoid receptor named CB₂ was identified in the human promyelocytic leukemic cell line HL60 (Munro *et al.* 1993). The distinction between these two receptors is based on differences in their amino acid sequence, signalling mechanisms, tissue distribution, and sensitivity to selective agonists and antagonists.

CB₁ and CB₂ receptors are G-protein-coupled receptors members of G_{i/o} family (G_{i1}, 2 and 3, and G_{o1} and 2), for review see (Howlett 2005). Both these receptors inhibit adenylylase in most cells via G_i, although CB₁ receptors can signal via G_s and stimulate adenylylase in some experimental models (Glass and Felder 1997; Maneuf and Brothie 1997; Calandra *et al.* 1999).

Activation of CB₁ and CB₂ cannabinoid receptors leads to phosphorylation and activation of p42/p44 mitogen-activated protein kinases (MAPK) (Bouaboula *et al.* 1995; Bouaboula *et al.* 1996), p38 MAPK (Derkinderen *et al.* 2001) and Jun N-terminal kinase (JNK) (Rueda *et al.* 2000) as signaling pathways to regulate nuclear transcription factors.

Evidence exists that CB₁ receptor via G_{i/o} inhibits N- and P/K-types of voltage-gated Ca²⁺ channels and stimulates A-type and inwardly rectifying potassium channels (Howlett 2005; Pertwee *et al.* 2010).

The CB₁ receptor possesses one or more allosteric sites and different ligands may enhance or inhibit the activation of this receptor by direct agonists (Price *et al.* 2005; Horswill *et al.* 2007; Navarro *et al.* 2009).

It is well known that CB₁ and CB₂ receptors are much more widely distributed than originally believed and that both receptor types can control central and peripheral functions. The CB₁ receptors are preferentially expressed in the central nervous system, being mainly distributed in brain areas associated with motor control, emotional responses, motivated behaviour and energy homeostasis, where they mediate inhibition of transmitter release (Matsuda *et al.* 1990). In the periphery, CB₁ receptors are expressed in the adipose tissue, pancreas, liver, gastrointestinal tract, skeletal muscles, heart and the reproduction system (Mackie 2008). While CB₂ receptors occur in the brain, pancreas, bone and adipose tissue, they are mainly expressed by immune cells (Munro *et al.* 1993; Patel *et al.* 2010).

Endocannabinoids

The discovery of CB₁ and CB₂ receptors opened the way to the identification of their cannabis-like endogenous ligands, named endocannabinoids. The *N*-arachidonylethanolamine (anandamide) was the first endocannabinoid to be discovered (Devane *et al.* 1992). Shortly after, it was observed that an already known endogenous metabolite, 2-arachidonoyl-glycerol (2-AG) also exhibits high affinity for cannabinoid receptors (Mechoulam *et al.* 1995; Sugiura *et al.* 1995). Meanwhile, other polyunsaturated fatty acids have also been proposed to be endocannabinoids including, *N*-dihomo- γ -linolenylethanolamine, *N*-docosatetraenylethanolamine, virodhamine, oleamide and *N*-arachidonoyl dopamine, but are less well-characterized (Di Marzo 2008; Pertwee *et al.* 2010). Palmitoylethanolamide (PEA), an endogenous lipid congener of anandamide, with anti-inflammatory and anti-nociceptive properties, has been postulated to act by enhancing anandamide effects but it also activates transient receptor potential vanilloid receptor type 1 (TRPV1) (Petrosino *et al.* 2010). Therefore, anandamide and 2-AG are still referred as the “major” endocannabinoids.

Anandamide and 2-AG have affinity for both CB₁ and CB₂ receptors with slightly greater affinity for CB₁ (Pertwee *et al.* 2010; Cluny *et al.* 2012). The

endocannabinoids also activate other receptors, such as the orphanized G-protein-coupled receptor-GPR55, 5-HT₃ receptors, opioid receptors, peroxisome proliferator-activated receptors (PPARs), TRPV1 (anandamide, but not 2-AG) and TRPV4 (both anandamide and 2-AG) (Pertwee *et al.* 2010; Cluny *et al.* 2012).

As lipids, endocannabinoids cannot be stored in vesicles, but are produced via several biosynthetic pathways, as needed (“on demand”) from membrane phospholipids in response to an increase in intracellular Ca²⁺ or after activation of G-protein-coupled receptors (Alger and Kim 2011; Cluny *et al.* 2012).

2-AG can be formed when Ca²⁺ stimulates phospholipase C which then transforms membrane phosphoinositides into DAG, from which 2-AG is liberated by DAG lipase (DGL) (Alger and Kim 2011). Alternatively DAG can be produced from phosphatidic acid, a reaction catalysed by either phospholipase A₂ or D (Alger and Kim 2011). Two isoforms of DGL have been cloned: DGL α and DGL β , but DGL α seems to be sufficient for most endocannabinoid signalling (Alger and Kim 2011).

There is no consensus as to which of the multiple pathways of anandamide synthesis is physiologically more relevant (Di Marzo 2009; Alger and Kim 2011; Cluny *et al.* 2012; Fowler 2012). *N*-Arachidonoyl-phosphatidylethanolamine (NArPE) is the major biosynthetic precursor of anandamide (Cadas *et al.* 1997; Di Marzo 2009). The enzyme that catalyses the direct conversion of NArPE to anandamide is known as *N*-acylphosphatidyl-ethanolamine specific phospholipase D (NAPE-PLD) (Di Marzo 2009; Cluny *et al.* 2012) but other routes of synthesis are described since NAPE-PLD “knock-out” do not exhibit reduced levels of anandamide in most tissues (Leung *et al.* 2006).

Endocannabinoids are produced on demand and are rapidly cleared by a process of cellular uptake that is incompletely characterized, followed by enzymatic metabolism. There is general consensus that the enzyme fatty acid amide hydrolase (FAAH) is the key-enzyme of the breakdown of anandamide, (Di Marzo 2009). On the other hand, besides the major contribution of monoacylglycerol lipase (MAGL) for 2-

AG metabolism, it may also be metabolized with a lesser extent by α/β hydrolase 6, α/β hydrolase 12 and FAAH (Di Marzo 2009; Alger and Kim 2011). While the searches for α/β hydrolases inhibitors are still in their beginning, a growing number of selective and potent inhibitors are now available to inhibit FAAH and MAGL activities (Petrosino and Di Marzo 2010; Feledziak *et al.* 2012).

Enzymes of the arachidonic acid cascade such as COX-2 and lipoxygenases are also alternative pathways for 2-AG and anandamide metabolism, leading to the formation of active metabolites capable of acting at the cannabinoid, TRPV and PPAR receptors (Duggan *et al.* 2011; Vecchio and Malkowski 2011; Cluny *et al.* 2012). The biological relevance of these reactions remains to be established.

Relevance of endocannabinoid system

In recent years, it becomes clear that the functions of endocannabinoid system are exerted in the whole organism and are not limited to the central nervous system (Di Marzo 2008; Pacher and Mechoulam 2011). To date, it has been documented that endocannabinoid system-regulated functions include: neuronal transmission, pain initiation, thermogenesis, appetite and energy metabolism, inflammatory and immune responses, bone remodelling, lipid metabolism, cardiovascular, respiratory and reproductive functions, hormone release, as well as cellular proliferation and apoptosis (Calignano *et al.* 1998; Di Marzo 2008; Pacher and Mechoulam 2011).

The endocannabinoid system is activated “on demand” and a pro-homeostatic effect has been claimed in different tissues. This “plasticity” of the endocannabinoid system is clearly observed in central nervous system, where it underlies adaptive responses to anxiety, chronic stress, neuronal damage and neuroinflammation (Bisogno and Di Marzo 2007; Moreira *et al.* 2008). The tissue localization of the cannabinoid receptors and metabolic enzymes also support this proposed pro-homeostatic strategy of action.

The expression of cannabinoid receptors and also the tissue and plasma levels of the “major” endocannabinoids undergo significant changes following physiological and pathological stimuli. However, for the same pathological condition, there are often reports of both positive and negative changes and of both protective and deleterious effects of endocannabinoid system activation (Di Marzo 2008). It is becoming increasingly evident that within a certain tissue, the endocannabinoid system may be affected in different ways by the same stressful stimulus according to the duration of this stimulus.

The endocannabinoid system is involved in various pathological conditions in central and peripheral tissues such as, neurodegenerative disorders, psychiatric conditions, cardiovascular disease, liver disorders, osteoporosis, inflammatory bowel disease, auto-immunity, cancer, obesity and metabolic syndrome (Di Marzo 2008; Moreira *et al.* 2008; Pacher and Mechoulam 2011).

Obesity and metabolic syndrome are well-known pathological conditions associated with endocannabinoid system dysregulation. (Maccarrone *et al.* 2010; Cluny *et al.* 2012). Endocannabinoid system plays a major role in the regulation of energy homeostasis and is generally upregulated in chronic overeating (Maccarrone *et al.* 2010). The endocannabinoid plasma levels and FAAH activity in subcutaneous adipocytes are positively correlated with body mass index (BMI) (Cable *et al.* 2011) and in obesity elevated endocannabinoid plasma levels were associated with coronary circulatory dysfunction (Quercioli *et al.* 2011). It was demonstrated that endocannabinoids also play a key role in the development of fatty liver in response to high fat diets or chronic alcohol intake (Jeong *et al.* 2008). Furthermore hypothalamic endocannabinoids seem to be part of the neural circuitry involved in the modulating effects of leptin on energy homeostasis.

Strategies for manipulating the endocannabinoid system for therapeutic reasons will require a thorough understanding of the roles of the different endocannabinoids, the stimuli that mobilize them and their sources and metabolism. Regulating

endocannabinoid levels *in vivo* represents an interesting therapeutic perspective. Several inhibitors of 2-AG and anandamide metabolizing enzymes have attracted growing interest as potential therapeutic drugs. Particularly, the FAAH inhibitors have demonstrated benefit in animal models of several disorders, including pain, anxiety and inflammatory bowel diseases, as well as against proliferation and migration of cancer cells (Clapper *et al.* 2010; Petrosino and Di Marzo 2010).

Cannabinoid compounds have also been proposed as promising therapeutic agents in multiple sclerosis given their capability to alleviate symptoms (e.g., spasticity, pain) and reduction of inflammatory events by the activation of CB₁ receptors (de Lago *et al.* 2012). CB₁ receptor antagonists have been explored, and found to be effective, as therapeutic agents for obesity and related cardiometabolic problems. However, the use of rimonabant, the first marketed CB₁ receptor antagonist, has been suspended due to its anxiogenic and depressogenic effects (Kirilly *et al.* 2011).

Endocannabinoid system in the skin and appendages

The skin and its appendages (hair follicle and sebaceous gland) function as a “neuro-immuno-endocrine” organ. Indeed, almost all skin cell types are capable of producing and releasing pro and/or anti-inflammatory mediators, hormones, growth factors, neuropeptides and vasoactive substances that can exert paracrine or autocrine regulation of skin cells functions (Roosterman *et al.* 2006).

Recent evidences suggest that endocannabinoid system has an important role in this complex cutaneous network (Kupczyk *et al.* 2009).

Anandamide and 2-AG are detectable in rodent skin (Calignano *et al.* 1998; Karsak *et al.* 2007) and the distribution and expression of CB₁ receptors was uniformly found in human skin biopsies taken from different body sites (Stander *et al.* 2005).

CB₁ and CB₂ receptors were predominantly expressed on cutaneous nerves (Stander *et al.* 2005) and it was proposed that cannabinoid receptors present in the skin might act as cutaneous nociceptors (Ibrahim *et al.* 2005; Khasabova *et al.* 2008).

In normal skin, CB₁ and CB₂ receptors were mostly present in suprabasal layers of the epidermis and in skin appendages like epithelial cells of hair follicles and sebocytes (Casanova *et al.* 2003; Stander *et al.* 2005).

There is evidence that anandamide and 2-AG and both cannabinoid receptors are present on human keratinocytes, which also express the main enzymes involved in anandamide synthesis (NAPE-PLD) and degradation (FAAH) (Maccarrone *et al.* 2003; Ibrahim *et al.* 2005).

Anandamide in a CB₁ receptor-dependent manner inhibits keratinocyte differentiation by transcriptional downregulation of keratin 1, keratin 5, involucrin and transglutaminase 5 (Paradisi *et al.* 2008). These effects were mediated by an increase in DNA methylation through MAPK-dependent pathways (p38, p42/44) (Paradisi *et al.* 2008).

Published data on effects of cannabinoids on regulation of keratinocytes cell death are conflicting. Wilkinson *et al.* (Wilkinson and Williamson 2007) found that phytocannabinoids such as Δ^9 -tetrahydrocannabinol as well as synthetic cannabinoid agonists inhibited growth of cultured transformed human epidermal keratinocytes (HPV-16E6/E7), yet these effects were CB₁ and CB₂ independent. In other study, activation of both cannabinoid receptors by synthetic agonists induced the apoptotic death of tumorigenic epidermal cells whereas the viability of non-tumorigenic human (HaCat cells) and murine (MCA3D) keratinocytes remained unaffected (Casanova *et al.* 2003). In contrast, recent data on CB₁ and CB₂ knockout mice suggest that cannabinoid receptors and the related signalling pathways might be involved in skin cancer development (Zheng *et al.* 2008). The effect of cannabinoids in keratinocytes death needs further investigation since manipulation of this pathway could become a

useful adjunct treatment option in hyperproliferative dermatoses such as psoriasis or keratinocytes-derived skin cancers.

Despite this well documented endocannabinoid system in human keratinocytes the role of endocannabinoids in melanocytes has not yet been studied. There is one study (Blazquez *et al.* 2006) demonstrating that human and mouse melanoma cells contain cannabinoid CB₁ and CB₂ receptors. *In vitro* experiments on A353 and Meljuso melanoma cell lines, revealed that cannabinoids significantly decrease the number of viable melanoma cells by inducing apoptosis and that this effect was prevented by selective cannabinoid receptors antagonists (Blazquez *et al.* 2006). Interestingly proliferation of normal melanocyte cell lines was not inhibited (Blazquez *et al.* 2006). Furthermore, in the same paper, it was documented that CB₂ receptor agonists inhibit melanoma progression and metastatic spreading in the mouse (Blazquez *et al.* 2006).

In human scalp, it was demonstrated that hair follicles are sources of endocannabinoids and express CB₁ receptors. CB₁ activation by anandamide inhibited hair growth and induced apoptosis-driven premature hair follicles regression (catagen) *in vitro* (Telek *et al.* 2007). Hair follicles are most common arranged in pilosebaceous units, which display another adnexal structure of the human skin, the sebaceous gland. Dobrosi *et al.* (Dobrosi *et al.* 2008) by using a human sebaceous gland-derived cell line demonstrated that anandamide and 2-AG are produced by epithelial cells of the sebaceous glands (sebocytes) and both via CB₂ receptor and MAPK pathway, dose-dependently induce lipid production and cell death (Dobrosi *et al.* 2008). Moreover, endocannabinoids also upregulated the expression of key genes involved in lipid synthesis, suggesting that CB₂ ligands may be exploited in the management of sebaceous glands dysfunctions (Dobrosi *et al.* 2008).

Since the original discovery of the CB₂ receptors in immune cells, much evidence suggest that the endocannabinoid system has important immune modulator effects during inflammation (Klein 2005). Karsak *et al.* (Karsak *et al.* 2007) observed

an increase in cutaneous nickel-induced allergic responses in CB₁ and CB₂ knockout mice as well as in the presence of cannabinoid receptors antagonists. In contrast FAAH-deficient mice displayed reduced allergic responses in the skin (Karsak *et al.* 2007). By using an animal model of induced allergic contact dermatitis it was also demonstrated that after allergen exposure, the cutaneous levels of 2-AG and anandamide significantly increased and that CB₁ receptor RNA_m was downregulated while CB₂ receptor mRNA was upregulated (Karsak *et al.* 2007). Taken together these data suggest a protective role of the endocannabinoid system in contact allergic dermatitis (Karsak *et al.* 2007). A protective role of PEA against inflammation was also observed in the same animal model of contact allergic dermatitis and in a keratinocyte cell line (HaCat) (Petrosino *et al.* 2009).

Endocannabinoid signals involved in skin inflammation are complex and remain poorly understood. Overall, anandamide appears to mediate an anti-inflammatory effect since inhibition of its degradation significantly reduced inflammation (Karsak *et al.* 2007) and, on the other hand, there is growing evidence suggesting that 2-AG modulates the inflammatory response by acting on the CB₂ receptors (Oka *et al.* 2006). By using different animal models for acute and chronic dermatitis, Oka *et al.* (Oka *et al.* 2006) reported elevated 2-AG levels in the affected skin and that CB₂ (but not CB₁) antagonists markedly attenuated cutaneous inflammation. Likewise, others reported a decrease in the cutaneous inflammation of CB₂ receptor deficient mice (Ueda *et al.* 2007).

In addition the endocannabinoid system has a crucial role in the control of skin-derived sensory phenomena as pain and itch.

Despite significant research on the role of endocannabinoid signaling in keratinocytes and pilosebaceous units the potential role of endocannabinoid system in melanogenesis and the influence of UVR in cutaneous endocannabinoid tone are not yet elucidated.

Introduction

Annex 1 | *Mechanisms regulating melanogenesis*

An Bras Dermatol 2012 in press

Annex 2 | *Phototherapy and photopheresis: old and new indications*

Exp Review Dermatol 2011;6(6):613-623

Mechanisms regulating melanogenesis

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Abstract

The skin pigmentation is an important human phenotypic trait but despite many efforts its regulation is still not fully understood. The pigment melanin is produced in melanosomes by melanocytes in a complex process named melanogenesis. The melanocyte interacts with endocrine, immune, inflammatory and central nervous systems and its activity is also regulated by extrinsic factors such as ultraviolet radiation and drugs. We review current understanding of intrinsic and extrinsic factors regulating skin pigmentation, melanogenesis steps and the known related gene defects. We focus on melanocyte-keratinocyte interaction, enzymatic components of melanosomes, activation of melanocortin receptor type1 (MC1-R) by proopiomelanocortin (POMC) cleavage products (melanocyte stimulating and adrenocorticotrophic hormones) and mechanisms of ultraviolet-induced skin pigmentation. The identification and understanding of the melanogenesis mechanisms facilitate the knowledge of the pathogenesis of pigmentation disorders and the development of potential therapeutic options.

Introduction

The skin has epidermal units that are responsible for melanin production and distribution, in a process called melanogenesis. These units are composed by a melanocyte surrounded by keratinocytes and they are regulated by a closed paracrine system. Melanin is the main responsible for skin, hair and eyes pigmentation and besides defining an important human phenotypic trait, it has a critical role in photoprotection due to its ability to absorb ultraviolet radiation (UVR) [1-3]. The Fitzpatrick system is the most commonly used system to distinguish the diversity of cutaneous pigmentation phenotypes. It characterizes six phototypes (I-VI), by grading the erythema and the acquired pigmentation after exposure to UVR [1, 4]. Constitutive pigmentation reflects the genetically determined level of melanin and can be changed by several regulator factors [3, 5]. These factors may be intrinsic (released by keratinocytes and fibroblasts, endocrine, inflammatory and neuronal cells) or extrinsic (UVR and drugs) [3, 6]. The melanogenesis is a complex process with different steps and when disturbed may determine different types of pigmentation defects, which are classified as hypo or hyperpigmentation and may occur with or without altered melanocytes number [1, 2, 7]. There are several dermatoses associated with pigmentation defects which can be congenital or acquired, permanent or temporary and restricted to the skin or systemic [7]. Since these dermatoses have an important impact on patient's quality of life and their treatment can be unsatisfactory, the pharmaceutical and cosmetic industries have been continuously seeking for new solutions [8, 9]. The understanding of the melanogenesis mechanisms helps us to explain the pigmentation defects observed in genodermatoses

and allows the development of potential therapeutic strategies [3, 10]. In this review we describe the intrinsic and extrinsic factors that regulate human skin pigmentation, focusing on melanogenesis mechanisms and related genodermatoses.

Term	Abbreviation
ACTH	Adrenocorticotrophic hormone
α -MSH	Melanocyte stimulating hormone
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
c-kit	Mast cell growth factor
CREB	cAMP response element
ET	Endothelin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL	Interleukin
IP3/DAG	Inositol triphosphate / diacylglycerol
MAP	Mitogenic activated protein
MATP	Membrane-associated transporter protein
MC1-R	Melanocortin receptor type 1
MITF	Microphthalmia-associated transcription factor
NGF	Neuronal growth factor
NO	Nitric oxide
PG	Prostaglandin
PKA	Protein kinase A
PKC- β	Protein kinase C- β
POMC	Proopiomelanocortin
ROS	Reactive oxygen species
SCF	Stem cell factor
TNF- α	Tumor necrosis factor α
TRP	Tyrosinase-related protein
UVR	Ultraviolet radiation

Methods

This review includes research articles which are supported by PubMed (basic electronic bibliographic database), are written in English and are available over the Internet by using search terms as: *Human melanogenesis; Melanocyte Biology and Pigmentation Skin; Pigmentation disorders*.

Mechanisms of melanogenesis

Steps in melanogenesis

Melanocytes originate in neural crest **melanoblasts** that, after the closure of the neural tube, migrate to different destinations, including the basal layer of the epidermis and hair follicles [3, 11, 12]. The migration, proliferation and differentiation into melanin producing cells depend on mediators produced by cells of the dorsal neural tube, ectoderm and keratinocytes, such as, respectively, the family of glycoproteins WNT, endothelin 3 (EDN3) and stem cell factor (SCF) that binds the c-Kit tyrosine kinase receptor on the melanocyte and melanoblast [3, 12]. The bone morphogenic proteins antagonize these events, and its expression is reduced in melanocytes migration. The **Piebaldism** (Table 2), a genodermatose with depigmented macules, is caused by mutations in the c-kit and SCF genes [3, 10, 12].

The melanin synthesis occurs in **melanosomes**, lysosome-related organelles (LROs), whose defects are responsible for **Chediak-Higashi Syndrome** and **Hermansky-Pudlak Syndrome** (Table 2), diseases with cutaneous hypopigmentation and systemic manifestations [6, 10, 11, 13].

The key proteins involved in skin pigmentation, such as the components of the fibrillar matrix that binds to melanin (glycoprotein Pmel17) and melanogenic enzymes, are localized in melanosomes. In these organelles, across four maturation stages, the structural matrix is arranged, the enzyme tyrosinase is acquired and melanin is synthesized [2, 3, 6, 13]. The acquisition of melanogenic enzymes is regulated by a membrane-associated transporter protein (MATP) and mutations of the respective gene determine **Oculocutaneous Albinism type 4** [3, 6, 10]. When melanin synthesis is completed, melanosomes move bi-directionally from the perinuclear area towards melanocyte dendrites, in a movement controlled by microtubule proteins (kinesin, dynein). This transport ends with melanosome linkage to actin filament by a complex formed by myosin Va, Rab27a and melanophilin (mlph) [2]. Mutations in the corresponding genes determine various forms of **Griscelli Syndrome** (Table 2) [6, 10]. An increase of intramelanosomal pH from 5 to 6.8, which depends on the proton pump p-protein in melanosomes membrane, is needed to full maturation of melanosomes [14]. The importance of this step is supported in on one hand by **Oculocutaneous Albinism type 2** (Table 2), a disease that is caused by the loss of functional p-protein, and in another hand by the lower response to repigmentation treatments observed in **Vitiligo**

patients who are also treated with proton pump inhibitors [6, 12, 14].

In the epidermis, each melanocyte interacts through dendrites with 30 to 40 keratinocytes allowing the **transfer of mature melanosomes into the cytoplasm of keratinocytes** positioned strategically over nuclei [1, 11]. This transfer is not fully understood and different mechanisms are described: exocytosis, citophagocytosis, fusion of plasma membranes and transfer by membrane vesicles [2].

Phenotypic diversity of pigmentation and types of melanin

The phenotypic pigmentation diversity is not due to a variation in melanocytes number, which is relatively constant in different ethnic groups, but to the size and number of melanosomes, the amount and type of melanin and its transfer and distribution in the keratinocytes [1, 3, 11]. The melanosomes of dark-skinned individuals are larger, more numerous and elongated resulting in a delayed degradation in the keratinocytes and consequently in an increased visible pigmentation [3, 11, 13]. These differences in melanosomes are present at birth and are not determined by extrinsic factors such as UVR [3].

There are two types of melanin (Figure 1): **eumelanin** - brown-black or dark insoluble polymer, and **phaeomelanin** - red-yellow soluble polymer formed by the conjugation of cysteine or glutathione [11, 13, 15]. The eumelanin is the major type in individuals with dark skin and hair and is more efficient in photoprotection. The pheomelanin is predominantly found in individuals with red hair and phototypes I and II, in which skin tumors are more common [5, 11].

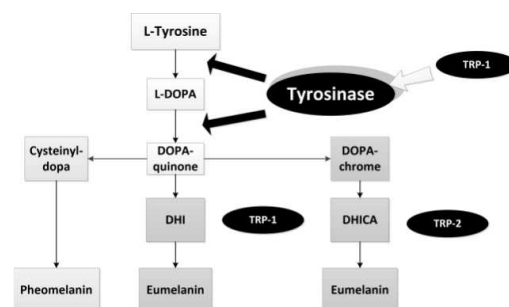


Figure 1 – Synthesis of two types of melanin with representation of functions of the major enzymes involved.

Enzymes of melanogenesis

Tyrosinase is a glycoprotein located in the melanosomal membrane, with an internal, a transmembrane and a cytoplasmic domain. It is a copper-dependent enzyme that catalyzes the

Table 2 – Genodermatoses with hypopigmentation or depigmentation. *LYST* (gene of factor that regulates lysosomes transport); *OCA2* (gene of p-protein pumping proton), *TR* (gene of tyrosinase).

Genodermatoses	Defect melanogenesis	in	Affected gene	Heredity; clinical characteristics
Piebaldism	Melanoblasts proliferation migration	and	C-KIT, SCF	AD; depigmented skin macules and white forelock
Waardenburg Syndrome (WS)	Melanoblasts proliferation migration	and	WS1 and WS3 : <i>PAX3</i> WS2 : <i>MITF</i> , <i>SOX10</i> WS4 : <i>SOX10</i> , <i>EDN3</i>	AD; depigmented skin macules and white forelock, heterochromia of the iris, deafness
Tietz Syndrome	Melanoblasts proliferation migration	and	<i>MITF</i>	AD; hypopigmentation and deafness
Oculocutaneous Albinism (OCA)	Melanin synthesis		OCA1 : <i>TR</i> OCA2 : <i>OCA2</i> (p gene) OCA3 : <i>TRP1</i> OCA4 : <i>MATP</i>	AR; pink skin, white hair, blonde, brown, red pupils, reduced visual acuity, nystagmus, photophobia
Menkes Syndrome	Melanin synthesis		<i>ATP7A</i>	X-linked recessive; sparse scalp hair, spleen, white or gray, neurological disorders
Chediak-Higashi Syndrome	Melanosomes synthesis		<i>LYST</i>	AR; silvery sheen of the skin and hair, hypopigmentation of the iris, neurological disorders, ocular albinism, immunodeficiency, pancytopenia
Hermansky-Pudlak Syndrome (HPS)	Melanosomes synthesis		<i>HPS</i>	AR; white patches on the skin and ocular albinism, bleeding tendency
Griselli Syndrome (GS)	Melanosomes transfer		GS1 : <i>MYO5A</i> GS2 , <i>RAB27A</i> GS3 : <i>HPLM</i>	AR; skin and hair hypopigmentation, ocular albinism, neurological disorders

conversion of L-tyrosine into L-DOPA, the rate-limiting step in the melanin synthesis (Figure 1) [2, 14, 15]. Mutations that inactivate this enzyme are responsible for the most severe form of Albinism, **Oculocutaneous Albinism type 1** (Table 2) [6]. The cytoplasmic domain participates in the transport of the enzyme from the nucleus to the melanosomes. The internal domain contains the catalytic region (approximately 90% of the protein) with histidine residues, where the copper ions binds [2]. Mutations in the copper carrier (*ATP7A*) result in **Menkes Disease** (Table 2) [6]. If copper is oxidized, the enzyme is inactivated and can be activated by electrons donors such as L-DOPA, ascorbic acid, superoxide anion, and possibly nitric oxide (NO) [14, 15]. Due to the fact that this enzyme can use superoxide anion as a substrate for melanogenesis it may protect melanocytes from reactive oxygen species (ROS) [11, 16]. The phosphorylation of two serine residues from cytoplasmic domain by the protein kinase C- β (PKC- β) is also important for tyrosinase activation [17].

The **tyrosine hydroxylase isoform I (THI)** is present in melanosome's membrane adjacent to tyrosinase and catalyzes the conversion of L-tyrosine into L-DOPA promoting the activation of tyrosinase.

In cytosol, the **phenylalanine hydroxylase (PAH)**,

6BH4 (6-tetrahydrobiopterin) cofactor dependent, catalyzes the conversion of L-phenylalanine to L-tyrosine, the tyrosinase substrate, thus also promoting its activation [14, 15]. Schallreuter and colleagues [14], underlining the central role of tyrosinase, consider that these three enzymes are required for the beginning of melanogenesis.

Two proteins similar to tyrosinase (40% homologous amino acids), **tyrosinase-related protein-1 (TRP-1)** and **tyrosinase-related protein-2 (TRP-2)**, are also present in the membrane of melanosomes. Although its precise role is not yet clarified, it is possible that the TRP-1 has a role in the activation and stabilization of tyrosinase, melanosomes synthesis, increased ratio eumelanin/pheomelanin (Figure 1) and against oxidative stress by its peroxidase effect [2, 15]. The results of Jimbow and colleagues [18] suggest that the premature death of melanocytes in **Vitiligo** is related to an increased sensitivity to oxidative stress by changes in the TRP-1.

Mutations of TRP-1, present in **Oculocutaneous Albinism type 3** (Table 2), result in skin and hair hypopigmentation [6]. TRP-2 acts as a dopachrome tautomerase (Figure 1) and like tyrosinase requires a metal ion for its activity - zinc instead of copper [2, 14, 15]. Figure 1 shows the synthesis of the two types of melanin and the functions of the major enzymes involved.

Table 3 – Effects of paracrine factors secreted by keratinocytes after UVR exposure. (adapted from Fitzpatrick's Dermatology in General Medicine [12]). ACTH (adrenocorticotrophic hormone), α -MSH (melanocyte stimulating hormone), bFGF (basic fibroblast growth factor), BMP-4 (bone morphogenic protein-4), ET-1 (endothelin-1), GM-CSF (granulocyte-macrophage colony-stimulating factor), IL-1 (interleukin 1), NO (nitric oxide), NGF (nerve growth factor); PGE2/PGF2 α (prostaglandin E2 and F2 α), TNF- α (tumor necrosis factor- α).

	Melanocytes proliferation	Dendricity	Melanin synthesis	Melanosomes transfer	Survival / Cytoprotection
ACTH	↑		↑		↑
α -MSH	↑	↑	↑		↑
bFGF	↑↑				
ET-1	↑	↑	↑		
GM-CSF	↑		↑		
NO			↑		
NGF		↑			↑
PGE2/PGF2 α		↑	↑	↑	
IL-1	↓	↑	↓		
TNF- α			↓		
BMP-4			↓		

Melanocortin-receptor type 1 (MC1-R)

Melanocortin receptors belong to G-protein-receptors family. MC1-R predominates in melanocytes and its agonists include melanocyte stimulating hormone (α -MSH) and adrenocorticotrophic hormone (ACTH), both cleavage products of proopiomelanocortin (POMC). POMC is cleaved by carboxypeptidase-1 in ACTH and β -lipotrofina and by carboxypeptidase-2 β in endorphin and ACTH. ACTH is fragmented in ACTH 1-17 and α -MSH. ACTH and α -MSH share the tetrapeptide His-Phe-Arg-Trp, which is essential to the melanotrophic activity. These peptides are the main intrinsic regulators of pigmentation, but its pituitary production is insufficient to stimulate melanogenesis, being keratinocytes and melanocytes the main responsible for its production on the skin [1, 5, 11, 13, 15, 16, 19]. **Addison's Disease** with high levels of ACTH, ACTH producing tumors (**Nelson Syndrome**) and cases of prolonged administration of this hormone are associated with hyperpigmentation, particularly in sun-exposed areas [1, 2, 15].

MC1-R genetic polymorphisms are responsible for ethnic differences of constitutive pigmentation and different responses to UVR exposure [2, 5, 11, 16]. In individuals with red hair and light skin there is a high incidence of MC1-R mutations that may be responsible for a decreased response to α -MSH, resulting in a decreased eumelanogenesis and reduced pigmentation induced by UVR exposure [11]. The *Agouti* signaling protein, although poorly documented, is the only known antagonist of MC1-R, competing with α -MSH and therefore stimulating pheomelanogenesis. MC1-R activation

by POMC peptides stimulates the accumulation of eumelanin instead of pheomelanin.

MC1-R agonists activate the adenylate cyclase enzyme, increasing intracellular cAMP and activating protein kinase A (PKA). PKA phosphorylates CREB (cAMP response element), which acts as a transcription factor in several genes, including the microftalmia-associated transcription factor (MITF). MITF in its phosphorylated active form regulates the expression of melanogenic enzymes promoting eumelanogenesis [2, 5, 11, 13, 15]. Its phosphorylation depends on kinases of mitogenic activated protein (MAP) whose activity is induced by the binding of keratinocyte-produced-SCF to the c-kit tyrosine kinase receptor [11, 12]. In addition to the CREB, the expression of the protein MITF is regulated by other transcription factors and mediators produced by keratinocytes and fibroblasts [6, 20]. Moreover, the protein MITF also regulates the expression of the Rab27a protein important in melanosome transport, Pmel17 protein of the melanosome matrix and of an anti-apoptotic protein (bcl-2) of melanocytes often expressed on melanoma [2, 10, 12, 20]. MITF gene mutations are responsible for **Waardenburg Syndrome type 2** with cutaneous and iris hypopigmentation, and **Tietz Syndrome** (Table 2) with hypopigmentation and deafness [1, 10, 20].

Intrinsic regulation of skin pigmentation

Melanocytes produce POMC peptides, cytokines, NO, prostaglandins and leukotrienes, which act through an autocrine or paracrine way on keratinocytes, and are involved in immune and inflammatory responses. Keratinocytes also produce several factors in response to UVR

exposure, with paracrine action on melanocytes, that may stimulate or inhibit melanogenesis (Table 3) [2, 6, 11, 12].

Our group investigated the role of the recently described cutaneous endocannabinoid system in melanogenesis and we demonstrated that UVR also activates endocannabinoid production by keratinocytes and that a paracrine cannabinoid receptor type 1-mediated endocannabinoid signaling negatively regulates melanin synthesis [21].

Despite POMC/MC1-R/cAMP being the main pathway, there are other melanocyte receptors associated with adenyliclase and cAMP production such as muscarinic receptors and α and β estrogen receptors [6]. The increase in estrogen levels during pregnancy can cause hyperpigmentation (melasma, areola hyperpigmentation and *line nigricans*). Catecholamines may be produced by keratinocytes from L-DOPA, the melanin precursor, and can bind to the melanocyte $\alpha 1$ and $\beta 2$ adrenergic receptors stimulating melanogenesis via cAMP pathway and PKC- β [15, 22]. This redundancy of cAMP production reveals the importance of this second messenger in melanogenesis. However, norepinephrine / $\alpha 1$ adrenergic receptor, ACTH 1-17/MC1-R can also activate the pathway inositol triphosphate / diacylglycerol (IP3/DAG), which promotes the release of calcium in the cytoplasm of melanocytes [2, 14, 22]. DAG is important for the activation of PKC- β , which phosphorylates tyrosinase, and can also be released from melanocyte by UVR action in the lipid membrane [3, 14, 17]. Figure 3 illustrates some of the different pathways, receptors, second messengers and melanogenic enzymes involved in melanogenesis.

Extrinsic regulation of skin pigmentation by ultraviolet radiation (UVR)

UVR is the most important extrinsic factor in the regulation of melanogenesis. It is the main stimulus for induced or acquired pigmentation, known as "tanning" [1, 2, 6, 11].

There are two types of induced pigmentation, which depends on genetic factors and are more evident in individuals with dark skin and hair [3, 23]. The **immediate pigmentation** which appears 5-10 minutes after exposure to UVR, disappears in minutes or days later, is largely due to UVA and is not dependent on the increased melanin synthesis but on the oxidation of melanin pre-existing and redistribution of melanosomes to the epidermal upper layers. The **delayed pigmentation** which occurs 3-4 days after exposure to UVR, disappears in weeks, is due to the UVA and mainly to UVB radiation and results from an increased level of

epidermal melanin, particularly eumelanin, providing photoprotection [3, 12, 13, 16, 23].

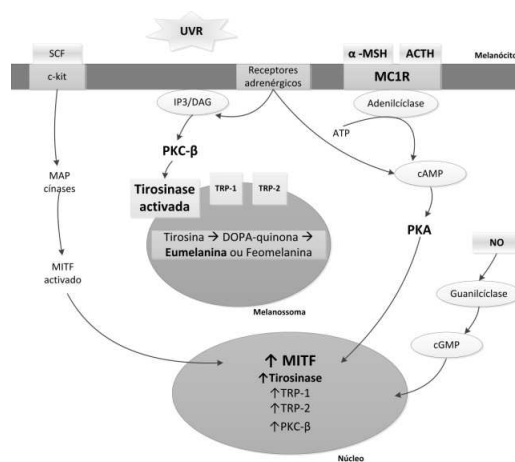


Figura.2

Figure 2 – Melanocyte role with representation of different signaling pathways regulating melanogenesis: activation factors, receptors, second messengers and melanogenic enzymes.

By one hand, UVR increases: proliferation and / or recruitment of melanocytes, the number of dendrites and the transfer of melanosomes to a supranuclear location on the keratinocytes for DNA photoprotection. On the other hand, the expression of POMC peptides, MC1-R and melanogenesis enzymes increases in keratinocytes and melanocytes respectively [11, 13, 16, 23].

DNA, the main cellular chromophore, directly absorbs UVR with the formation of thymine dimers and other pyrimidine derivatives and defects in DNA repair increase the risk of skin cancer [1-3, 5]. The key observation that the UVR spectra for producing a delayed tan [24] and for induction of thymine dimers following UVR [25] were virtually identical and within the UVB range suggested for the first time a cause effect relation between DNA damage and melanogenesis. In the last decade, it was recognized that the tumor-suppressor protein p53 is a transcription factor that plays a pivotal role in the tanning response after UVB-induced DNA damage [26]. Using an elegant mouse model in which UVR causes tanning it was demonstrated that α -MSH production is regulated in keratinocytes by p53 via a p53 consensus sequence in the POMC gene promoter [27]. Furthermore, Eller and colleagues [28] demonstrated in vitro that small fragments of DNA induced pigmentation by increasing tyrosinase expression and activity and tumor suppressor protein p53 levels [1]. Even in the absence of keratinocytes, there is a strong melanogenic response to UVR mediated by p53 in

human melanocytes and melanoma cells *in vitro* [2, 26], which can be explained by the fact that p53 regulates in melanocytes the transcription of the hepatocyte nuclear factor 1 α (HNF-1 α), that is a tyrosinase transcription factor [14].

The UVR also enhances reactive oxygen species (ROS) formation in keratinocytes and melanocytes, with consequent DNA damage [3, 5].

Regardless of the specific mechanisms, the acquired pigmentation is part of the adaptive response of skin, mediated by p53, to DNA damage caused by exposure to UVR and will provide skin protection to future exposures [2, 26].

It has been shown that plasma membrane lipids are also affected by UVR to release membrane-associated diacylglycerol (DAG) which activates PKC- β that in turn activates tyrosinase resulting in stimulation of melanogenesis [14].

An elderly individual, depending on the constitutive pigmentation and the cumulative UVR dose, may have hyperpigmented lesions (solar lentigines) that indicate photoaging. This can be explained by the fact that aged melanocytes possess an enhanced functional activity after years of cumulative UVR exposure. However with aging there is also a decrease in the number of functional melanocytes [3]. The eumelanin acts as a natural sunscreen against photoaging and photocarcinogenesis, in part by reducing ROS and increasing repair of DNA damage [5, 13, 15].

Conclusion

The melanocytes are responsible for the cutaneous synthesis and distribution of melanin, an essential pigment for photoprotection. This process named melanogenesis involves different stages since melanocyte embryogenesis to the melanosomes transfer to neighbor keratinocytes. The importance of each of these stages and their mechanisms is evident in the clinical genetic defects (genodermatoses with depigmentation or hypopigmentation). The identification of these defects has contributed to a better understanding of the melanocytes biology and the melanogenesis regulation. The melanogenesis study has revealed different interactions of melanocytes with other cells (including keratinocytes) and systems (CNS, immune, inflammatory, endocrine and endocannabinoid), and this raise the role of skin as a neuroendocrine organ.

Many factors are known as regulators of melanin synthesis, and we point UVR as an important extrinsic factor and α -MSH as an important intrinsic factor. The α -MSH exerts its effect mainly as an agonist of the MC1-R, whose genetic polymorphisms are part of the justification

for phenotypic diversity and differential response to UVR. In induced pigmentation, the UVR has a direct effect mediated by p53 tumor suppressor protein, and an indirectly effect by keratinocytes production of intrinsic factors, promoting eumelanin synthesis. The UVR is responsible for the "tanning", but is also associated with aging skin with hyperpigmented lesions and development of skin tumors.

The investigation of melanogenesis mechanisms is important in the understanding of the pigmentation defects and the consequent development of potential therapeutic agents. On the other hand, allows for the development of photoprotective measures, which reduce photoaging and photocarcinogenesis. This is an important area of research where still there is much to clarify and learn.

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EXPERT REVIEWS

Phototherapy and photopheresis: old and new indications

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The benefit of UV light in the treatment of skin disorders has been known for decades, but new discoveries have been made throughout the years since its first use, including new applications and different UV delivering devices. Broadband UVB has been largely replaced by narrowband UVB and, for selected uses, targeted narrowband UVB (excimer) is becoming more widely available. Psoralen UVA (photochemotherapy) remains the phototherapy of choice for certain disorders, but it has widely been replaced by narrowband UVB. UVA1 has currently a growing number of applications. Photopheresis is an extracorporeal form of photochemotherapy, mainly used in cutaneous T-cell lymphoma and graft-versus-host disease but also in autoimmune diseases. Herein, we review phototherapy's and photopheresis' applications after a brief summary on their mechanisms of action, dosing and administration, and safety issues.

KEYWORDS: atopic dermatitis • cutaneous T-cell lymphoma • graft-versus-host disease • photopheresis • phototherapy • psoriasis • PUVA therapy • scleroderma • UV light • vitiligo

Phototherapy

Phototherapy is the use of UV radiation in the treatment of skin disease [1]. Radiation within the UV spectrum can be divided by wavelength into UVC (200–280 nm), UVB (280–320 nm) and UVA (320–400 nm). Owing to the filtering effect of the earth's atmosphere, almost all UVC radiation and approximately 90% of the UVB is absorbed. Consequently, UVA makes up approximately 95% of the UV radiation that reaches the earth. With its longer wavelength, it can penetrate deep into the dermis, while UVB is absorbed in the epidermis. It is mainly UVB that burns the skin because it possesses much more energy than UVA (energy is inversely proportional to wavelength) [1].

Types of phototherapy include broadband-UVB (BB-UVB, 290–320 nm, peaks at 313 nm), narrowband-UVB (NB-UVB, 311–313 nm), UVA1 (340–400 nm, peaks at 365 nm), and combination therapy of psoralen plus UVA (PUVA photochemotherapy, 320–400 nm, peaks at 352 nm) [1,2]. Available psoralens include 8-methoxypsoralen (8-MOP; only form available in the USA), 5-methoxypsoralen (5-MOP; primarily used in Europe) and 4,5,8-trimethoxypsoralen [2]. The psoralen can be administered orally (5-MOP or 8-MOP) or

topically, either in the form of ointment, cream or lotion, either as bath-PUVA (8-MOP, 5-MOP or 4,5,8-trimethoxypsoralen) [2,3]. These treatment modalities are generally administered in a light booth or by hand and/or foot units [2].

Recently, the xenon chloride gas excimer offers a means for delivering larger fluences of 308 nm UVB selectively to the cutaneous lesions, with a decreased carcinogenic potential. Two systems have been developed: laser technology (known as excimer laser) and a new nonlaser technology (known as excimer light). The advantages of excimer light over the laser system are low operating costs and the fact that a large area can be treated quickly [4].

Photodynamic therapy and low-level light therapy are out of the scope of this review.

Mechanisms of action

The following mechanisms have been proposed to explain UV's efficacy in the treatment of skin diseases:

- Apoptosis of pathogenically relevant cells, including T-cell apoptosis, in the treatment of psoriasis, mycosis fungoides and atopic dermatitis [5,6], and mast-cell apoptosis, for pruritic skin disorders [7];

- Apoptosis of keratinocytes [8];
- Enhancement of melanocyte proliferation, for vitiligo [2];
- Decreased release of histamine from both basophils and mast cells, for histaminic disorders such as urticaria pigmentosa [2];
- Inhibition of ICAM-1 upregulation by keratinocytes in inflammatory diseases (which acts as counter-receptor for lymphocyte function-associated antigen-1 on the surface of leukocytes) [9];
- Downregulation of Th17 signaling pathway and decreased expression of IL-10 by keratinocytes, with a subsequent reduction of IFN- γ signaling and an anti-inflammatory effect [10,11]. Phototherapy has also been found to decrease the expression of IFN- γ -inducing cytokines IL-12, IL-18 and IL-23 [12];
- Increased expression of immunosuppressive cis-urocanic acid levels in the skin, resulting in suppression of cellular immune response and inhibition of antigen-presenting function of Langerhans cells [2,13].

In psoralen plus UVA (PUVA) photochemotherapy, psoralens photoconjugate to DNA with subsequent suppression of DNA synthesis and cell proliferation [14]. Also, reactive oxygen species production (derived from psoralens reacting with molecular oxygen) causes mitochondrial dysfunction and leads to apoptosis of Langerhans cells, keratinocytes and lymphocytes [2].

Dosing & administration

Before starting UVB phototherapy, the minimal erythema dose (MED) should ideally be determined (erythema 24 h after exposure) [2,15]. It is advised that the first treatment of NB-UVB equals 0.7 MED and that dose increments are made according to postirradiation erythema [2,15]. It is also possible to utilize Fitzpatrick skin type-dependent starting doses with subsequent fixed increments [15], and this is the approach we use in our center (Hospital de São João, Porto, Portugal). In the clearing phase, treatments are given 2–5-times weekly [15].

UVA-1 is further classified as low dose (20 mJ/cm²), medium dose (50–60 mJ/cm²) and high dose (120–130 J/cm²). Theoretically, full dose can be used as a starting dose because UVA1 does not induce erythema. However, to avoid any idiosyncratic reaction, it has been recommended to start at 20 mJ/cm², and then increase by 10 J/cm² per treatment until the full desired medium dose is reached. High-dose UVA-1 is no longer widely used [2].

In PUVA, the initial dose of UVA can either be determined by minimal phototoxicity dose (MPD; erythema 48–72 h after exposure) or by Fitzpatrick skin type [2]. In the first approach the initial dose should be 0.5–0.7 MPD for oral PUVA and 0.3 MPD for bath PUVA, with subsequent dose increments according to postirradiation erythema [15]. Similar to UVB dosing, UVA doses should be decreased if topical or systemic retinoids are added because they decrease stratum corneum thickness, thereby increasing phototoxicity [2]. In the clearing phase, irradiations are given 2–4-times weekly [15]. For oral PUVA, 0.6–0.8 mg/kg 8-MOP or 1.2–1.8 mg/kg 5-MOP is administered 1–3 h before

phototherapy [3]. Bath-PUVA consists of 15–30 min of whole-body immersion in solutions of 0.5–5.0 mg/l 8-MOP or 0.33 mg/l 4,5,8-trimethoxypsoralen [2,3]. Upon exiting the bath, the patient must receive the UVA dose within 30 min [2].

Safety

Most phototherapy regimens are very low risk for overall patient complications and morbidity [2].

UVB may cause acute phototoxicity, with erythema and blistering, beginning in the first 4–6 h after exposure and peaking at 12–24 h [2].

PUVA-induced erythema starts at 24–36 h, peaks at 48–72 h and may last 1 week or more. Avoidance of prolonged sun exposure, wearing UVA-absorbing sunglasses when outdoors, application of broad-spectrum sunscreens, and wearing of photoprotective clothing on the days of PUVA photochemotherapy are necessary measures to prevent significant phototoxicity. 8-MOP has a greater incidence of gastrointestinal side effects than 5-MOP. PUVA's itch is a subacute side effect of this treatment [2].

Both UVB and PUVA may also lead to tanning (requiring UV-dose increment), photo-onycholysis, melanonychia and friction blisters of treated areas. Photoaging is a long-term side effect, including PUVA lentigines. If proper protective eyewear is not worn, UVB-induced keratitis and PUVA-induced cataracts may also occur [2]. However, the recommendation for regular ophthalmologic evaluation of these patients is still controversial.

With respect to photocarcinogenesis, PUVA is associated with a dose-dependent increased risk of nonmelanoma skin cancers [16,17]. So far, no increase has been documented with NB-UVB [18], but long-term data are missing. Male genitalia should be shielded during every treatment session as they are particularly sensitive to the development of squamous cell carcinomas (SCCs) [2]. Increase in melanoma incidence following UV therapy is controversial [16,19].

Absolute and relative contraindications to phototherapy and photochemotherapy are shown in Boxes 1 & 2, as described by Sage and Lim [2]. UVB can be used in children as well as in pregnant and breast-feeding women, but long-term safety data are missing. Even though psoralens have never been proved to be teratogenic, they are not generally administered during pregnancy [2]. UVB is considered generally safer than PUVA in patients with skin types I and II, history of melanoma or nonmelanoma skin cancer, past history of x-ray therapy, arsenic exposure, or concomitant use of immunosuppressive agents [2]. Photosensitizing drugs usually react within the UVA spectrum and NB-UVB is thus generally safe in patients taking them [2]. UVB has been shown to be effective and safe in HIV patients [20], although a small study did show activation of the virus in the skin [21].

Indications

Psoriasis

UVB

Traditional BB-UVB radiation has been used for the treatment of psoriasis for more than 75 years. NB-UVB use was initially popularized in the UK and Europe in the mid-1980s, and became available in the USA approximately one decade later [22].

NB-UVB is superior to BB-UVB in respect to clearing and remission times [22], and it currently represents the phototherapeutic modality of choice for the treatment of psoriasis in Europe [23]. However, it is probably not as effective as PUVA, both in terms of clearing efficiency and duration of remission [15].

Patients with psoriasis who are compliant, motivated and adherent with instructions and follow-up examinations could, under dermatologist supervision, be considered appropriate candidates for home UVB therapy [22]. A recent multicenter, single-blind, randomized clinical trial demonstrated that home NB-UVB is just as effective as outpatient-administered NB-UVB, with superior patient quality of life [24]. We do not have experience with home phototherapy in our center.

In order to improve UVB's efficacy, it can be combined with other treatments, either topical or systemic.

Topical application of calcipotriol (after irradiation) or tazarotene [25] (before irradiation) may increase therapeutic efficacy of phototherapy alone [26]. By contrast, it seems that combining topical steroids adds little benefit to phototherapy [27]. Emmollients can increase UV transmission, increasing efficacy, but thick application of petrolatum and water-in-oil creams as well as salicylic acid and tar act as sunscreens and should be discouraged [28].

In regard to systemic drugs, retinoids increase efficacy and reduce the carcinogenic potential of UVB phototherapy [29,30]. Retinoids can be maintained after ceasing phototherapy to prevent recurrence. The short time combination of methotrexate with UVB therapy is of potential value because of the synergistic effects of these two therapies [22]. We find this combination particularly useful in patients with psoriatic arthritis in whom the articular disease is successfully managed with low-dose methotrexate, as phototherapy is not efficacious in treating arthritis. The combination of cyclosporine and UVB has not been studied extensively because of the increased risk of nonmelanoma skin cancer with cyclosporine monotherapy and it should generally be avoided [22]. Combination therapy of UVB with biologics has been reported to have a higher response rate than biologics alone, with a similar safety profile [22], but further investigation is needed. Potential concerns are increased phototoxicity and photocarcinogenesis.

Although UVB may be adequate for maintenance therapy [31], it is suggested to consider a rotational therapeutic approach in order to minimize a greater cumulative dose [32].

The first report on the use of the excimer laser to treat psoriasis goes back to 1997 [33]. Advantages include the possibility to treat exclusively the affected skin with higher doses (suprathymogenic) and lower number of treatments [34].

PUVA

Compared to oral 8-MOP, oral 5-MOP shows equal clearing rates with a similar number of exposures, but higher cumulative doses, lower incidence of pruritus or severe phototoxic reactions, and no nausea or vomiting [3].

Compared to oral 8-MOP, bath PUVA shows equal clearing rates with fewer exposures, similar or lower incidence of erythema and pruritus and no systemic intolerance such as nausea or vomiting [3].

Box 1. Contraindications to UV phototherapy.

Absolute contraindications

- Xeroderma pigmentosum
- Systemic lupus erythematosus
- Basal cell nevus syndrome

Relative contraindications

- Previous history of skin cancer
- Treatment of genital area
- Photosensitive disorders
- Contact photosensitive substances
- Photosensitizing medications with an action spectrum with the corresponding UVA or UVB wavelengths

Topical PUVA with psoralen creams, ointment or lotions is now used only for limited plaque psoriasis and for palmo-plantar disease [3].

With respect to combination therapy, the same considerations are valid as for UVB when it comes to topics. The combination of PUVA with systemic retinoids (RePUVA) is one of the most potent therapeutic regimens for psoriasis [15]. Methotrexate may also be considered for combination therapy, although the long-term risk of cutaneous malignancies, though never proved, should not be forgotten [15]. By contrast, concomitant use of cyclosporine has been recognized to enhance skin carcinogenesis [35]. Further investigation is needed to verify presumptive additive effects of biologics and PUVA [36].

PUVA can be considered for maintenance therapy but attention should be paid to long-term risks related to total cumulative phototoxic doses [3].

Pediatric population: In children, when topical treatment fails, NB-UVB phototherapy in combination with topical agents is generally the recommended treatment option before considering

Box 2. Contraindications to psoralen plus UVA.

Absolute contraindications

- Xeroderma pigmentosum
- Systemic lupus erythematosus
- Basal cell nevus syndrome
- Photosensitivity disorders
- Age less than 10 years
- Pregnant and breast-feeding patients
- History of melanoma

Relative contraindications

- Family history of melanoma
- History of dysplastic nevi or nonmelanoma skin cancers
- Photosensitizing medications
- Significant past UV exposure
- History of arsenic exposure, ionizing radiation, methotrexate, cyclosporine or tacrolimus

Caution

- Hepatic or renal insufficiency
- Patients 10–18 years of age

systemic therapy, including retinoids, methotrexate, cyclosporine and biological agents. The long-term risk of NB-UVB light in children is yet to be determined. PUVA can also be considered as a treatment option [37].

Cutaneous T-cell lymphoma (mycosis fungoides)

PUVA

The use of PUVA in cutaneous T-cell lymphoma (CTCL) was first reported in 1976 [38]. It is an excellent treatment option for the early stages of the disease (IA–IIA), achieving long-lasting free-disease intervals. It is also capable of reducing tumor burden in later stages (IIB–IVB), where it should be combined with other treatments, such as retinoids, bexarotene, INF- α 2a, local x-ray or systemic chemotherapy. Even with aggressive combination therapies, these patients experience multiple recurrences. Although effective in inducing remission in skin-confined disease, the effect of PUVA on the CTCL disease course and overall survival is yet to be determined. Currently there is no treatment option known to arrest progression of later stages [3,15,39].

After clearing, a maintenance phase is usually recommended, with two sessions per week for 1 month, followed by one session per week for another. Resolution should be confirmed with a biopsy. A follow-up is then performed, either without therapy, with one treatment per month or one treatment every other month. Relapses respond to therapy as well as initial lesions [3,15].

UVB

The first report of UVB phototherapy for CTCL goes back to 1982 [40]. NB-UVB can also induce high rates of complete remission in early disease [39]. Evidence is poor, however, regarding the question how NB-UVB compares with other treatments [39]. Studies comparing PUVA and NB-UVB have found them both effective in the treatment of CTCL [41,42]. NB-UVB and BB-UVB therapy have not been compared.

Excimer laser can also be considered a useful tool in the treatment of early-stage mycosis fungoides (MF) [43].

UVA1

UVA1 also appears to be effective [44], although the experience is limited. It offers advantages over PUVA in terms of side effects.

Pediatric population: In children, MF is rare and usually limited to early patch-stage disease. Both PUVA and UVB have been used [37].

Vitiligo

PUVA

PUVA has been used for longer than UVB in vitiligo and the available information for this therapy is greater than for UVB.

Patients should be aware that they will require months of treatment (100–200 treatments), with 70% of patients responding after 12–24 months. Responsiveness is defined as development of multiple perifollicular macules of repigmentation, or, in the case of small (<2 cm) lesions, contraction in size. However, if after 4–6 months or 30–50 treatments they do not respond, PUVA

should be terminated. Locations such as lips, distal dorsal hands, fingers and toes, palms, soles and nipples are very refractory to treatment, as are large areas with only white hairs. Stable disease (for at least a year) is usually easier to treat. Duration of disease before PUVA therapy does not affect response rate [3,15].

Completely repigmented areas can be stable for a decade or more without relapse. But if a lesion is not fully repigmented, reversal of acquired pigmentation may occur when treatment is discontinued [15].

KUVA and topical PUVA are alternatives to oral PUVA. KUVA uses khellin as a photosensitizer and this is not phototoxic. Topical PUVA may be suitable for few and small lesions [15]. UVA alone is of limited benefit [15].

UVB

Although the action spectrum for phototherapy of vitiligo is not known [15], UVB, in particular NB-UVB, is currently more often used than PUVA because it has been shown to be at least as effective and possibly safer [45,46]. As for PUVA, it is not very effective in certain anatomic areas (hands and digits, feet and toes) and treatment should be ceased if patients do not respond [23]. Excimer laser should be considered as a therapeutic option for localized lesions [47].

Pediatric population: In children, NB-UVB is the preferred phototherapeutic option owing to potential increased cancer risks associated with PUVA. However, PUVA can also be used in children older than 12 years of age [37].

Atopic dermatitis

Phototherapy of acute, severe atopic dermatitis

UVA1 phototherapy is a highly effective monotherapy for a limited period of time (ten to 15 exposures), particularly in cases of acute, severe atopic dermatitis [48]. It should not be used for maintenance therapy or for patients under the age of 18 years because its long-term effects are not known [23]. The effectiveness is dose-dependent, with high-dose UVA1 being more efficient than UVA/UVB phototherapy [49].

Phototherapy of chronic, moderate atopic dermatitis

BB-UVB, combined UVA/UVB, broadband UVA, low-dose UVA1 and, in particular, narrow-band UVB phototherapy are effective treatments in mild and moderate atopic dermatitis and they are generally used in combination regimens with topical steroids. They are relatively safe when used over extended periods of time for long-term management [23].

NB-UVB and UVA/UVB phototherapy are superior to BB-UVB, broadband UVA or low-dose UVA1 [23]. However, in a small number of children, BB-UVB may be less irritating and more effective than NB-UVB because it requires smaller doses to achieve minimal erythema and lymphocyte apoptosis [37]. The excimer laser can also be used to treat localized lesions of atopic dermatitis with faster clearance after fewer treatments compared with traditional NB-UVB or BB-UVB phototherapy [50].

After NB-UVB, the microbial population in atopic dermatitis patients is similar to control patients [51], but it is unknown

whether this translates clinically into reduced skin infections requiring antibiotics or decreased pruritus [37].

PUVA

PUVA can also be used to treat atopic dermatitis, but a high number of treatments are usually required and recurrences are high and rapid. Long-term treatment should be avoided [15].

Lichen planus

Oral and topical PUVA are therapeutic options for extensive lichen planus (LP) [3,15]. However, LP is usually more resistant than psoriasis, requiring more treatment sessions and higher cumulative UVA doses, with earlier relapses [3]. Combined PUVA-retinoid therapy may accelerate clearing [3]. Postinflammatory hyperpigmentation should be a potential concern. Besides, LP exacerbation during PUVA therapy has occurred in a few patients [15].

NB-UVB therapy can represent an effective option for refractory generalized LP according to a study of 16 patients in which complete response was achieved in 56.25% at 30 sessions and in 68.75% at 40 sessions, confirming the presence of significant correlation between session numbers and improvement of response, that had already been found in previous studies [52].

A study of 28 patients, 15 treated with oral PUVA and 13 with NB-UVB, concluded that even though oral PUVA produces a better initial clinical response rate, both oral PUVA and NB-UVB are effective treatments for LP, producing similar long-term outcomes.

Graft-versus-host disease

Currently, PUVA represents the mainstay for phototherapy of graft-versus-host disease (GVHD) [15]. It has been initially evaluated for lichenoid GVHD owing to clinical and histologic similarities with lichen planus, and it can clear or improve chronic and acute disease [53,54]. In sclerodermoid GVHD, results are controversial [3,15]. Effects may be systemic because mucosal lesions respond to treatment, but there is no improvement in other organs [3,15]. Patients should be followed-up for cutaneous tumors because there seems to be an overall increased risk of malignancies in bone marrow/stem cell recipients, independent of PUVA [3,15]. UVA1 therapy is also promising for the treatment of chronic GVHD, in either of its lichenoid or sclerodermoid variants [44,55]. UVB therapy has been described in a limited number of patients [15].

Pityriasis lichenoides

PUVA seems to be more effective than UVB in pityriasis lichenoides, especially in the acute form of the disease, where it has been recommended as the phototherapy of choice [15]. In pityriasis lichenoides chronic, PUVA may be reserved for cases of that are UVB resistant [15]. The experience with UVA1 is limited [56] and further investigation is needed.

Urticaria pigmentosa

PUVA

In cutaneous mastocytosis, PUVA therapy results in improvement of skin lesions [57], but in most patients these recur 5–8 months

after treatment discontinuation [3]. Response rate in relapses is though similar to initial lesions [3]. Systemic symptoms such as histamine-induced migraines and flushing also respond to PUVA [57]. PUVA therapy should be considered for patients in which disease is causing severe distress [3].

UVA1

Cutaneous mastocytosis has also been demonstrated to respond to UVA1 therapy, in its skin and systemic manifestations [58,59]. In a study of four patients, there were no recurrences more than 2 years after cessation of high-dose UVA1 therapy [59].

Photodermatoses

Phototherapy of patients with polymorphous light eruption (PMLE) induces tolerance to sunlight in a process known as hardening. Involved mechanisms include epidermal thickening, pigmentation and probable immunologic effects [3,15].

PUVA is the most effective preventive treatment in PMLE [3], but NB-UVB is currently the phototherapeutic option of choice, with recent studies showing that it is an effective alternative to PUVA [60].

With PUVA, 70% of patients develop hardening with a 3–4-week course treatment of 2–3 sessions a week in early spring. Patients remain protected if they have regular sunlight exposures. However, patients may remain protected for 2–3 months even after pigmentation has faded [3,15].

Patients with other photodermatoses, including actinic prurigo, hydroa vacciniforme and erythropoietic porphyria, may benefit from NB-UVB [61] or PUVA [3,15]. In solar urticaria, PUVA therapy appears to be the most effective therapy available [3]. Single UVA irradiation of quadrants of body surface hours before PUVA treatment may be useful when patients develop urticarial lesions in the beginning of treatment [3]. In therapy-resistant forms of solar urticaria, rush-hardening consists of multiple daily UVA irradiations at 1-h intervals, providing protection within 3 days [62]. Unlike PMLE, extended treatment may be required in solar urticaria and chronic actinic dermatitis [3].

Morphea, scleroderma & other sclerosing skin conditions

Morphea was shown to be effectively treated by UVA1 phototherapy in a dose-dependent manner [63]. Medium-dose UVA1 seems to be superior to low-dose UVA1 or NB-UVB [64,65]. The existing evidence indicates that medium–high dose UVA1 therapy delivered over 24–30 treatments can provide significant benefit to patients with morphea, particularly linear and plaque subtypes of disease [44]. UVA1 is likely ineffective in burned out atrophic lesions, deep morphea, Parry Romberg/facial hemiatrophy and eosinophilic fasciitis owing to the depth of the pathology, and systemic treatment should be considered as first-line therapy in these cases [44]. Little to no adverse effects have been reported with this therapy [44].

UVA1 might also be effective in treating patients with systemic sclerosis, studies to date showing improvement in hand/forearm disease, with a decreased hand score after treatment [66].

Localized scleroderma and pansclerotic morphea have also been successfully treated with bath PUVA and oral PUVA [67,68]. In our

center we have a good experience with topical PUVA for localized scleroderma and we consider this modality worth a try.

Lichen sclerosis has shown to be responsive to UVA1, in both genital and nongenital disease [69,70].

Other indications

UVB

NB-UVB was found to be an effective treatment in severe cases of seborrheic dermatitis, although flares may occasionally occur [71].

It has been shown that NB-UVB is effective in controlling uremic pruritus [72]. Although it can also be beneficial in other forms of pruritus, such as idiopathic or that associated with liver disease or diabetes [15], we have better results in uremic pruritus.

PUVA

The experience with PUVA in lymphomatoid papulosis is limited to a few successful anecdotal cases [73].

PUVA has been used in pityriasis rubra pilaris, but results are inconsistent [15,23].

Generalized granuloma annulare has been reported to clear completely, but long-term maintenance treatment was required to maintain remissions [74].

Uncontrolled studies of PUVA treatment for alopecia areata, using all types of PUVA (oral or topical psoralen, local or whole body UVA irradiation), report success rates of up to 60–65% [75], which are in accordance to our experience. Continued treatment might be needed however to maintain hair growth because relapse rates are high [75]. 308-nm excimer has also been reported as effective [76,77].

UVA1

Dyshidrotic hand eczema has been treated effectively with UVA1 therapy [78]. Long exposure to low-dose UVA1 might be beneficial for patients with systemic lupus erythematosus, with decreased fatigue, joint pain, malaise, stiffness and mouth ulcers, lasting over 3 years [79]. UVA1 therapy has also been shown to improve subacute cutaneous lupus erythematosus lesions, but not those seen in discoid lupus erythematosus [44]. A consensus statement by the North American Rheumatologic Dermatology Society has recently confirmed the potential beneficial effects of UVA1 phototherapy in systemic lupus erythematosus patients, but acknowledged that further investigation is needed to address its full benefit [44]. In TABLE 1, we summarize phototherapy's main clinical applications.

Table 1. Summary of the applications of phototherapy.

Condition	Standard of care	Other therapeutic options
Psoriasis	NBUVB	PUVA, Excimer
Cutaneous T-cell lymphoma (IA–IIA)	PUVA	NBUVB, Excimer, UVA1
Vitiligo	NBUVB	PUVA, Excimer
Atopic dermatitis: • Acute, severe • Chronic, mild	UVA1 NBUVB, UVA/UVB	
Lichen planus	PUVA	NBUVB
Lichenoid graft-versus-host disease	PUVA	UVA1, NBUVB
Pityriasis lichenoides	PUVA	NBUVB
Urticaria pigmentosa	PUVA	UVA1
Photodermatitis: • Polymorphic light eruption • Solar urticaria • Others	NBUVB PUVA NBUVB, PUVA	PUVA
Morphea, systemic sclerosis	UVA1	PUVA
Lichen sclerosis	UVA1	
Other conditions: • Seborrheic dermatitis • Uremic pruritus • Lymphomatoid papulosis • Pityriasis rubra pilaris • Granuloma annulare • Alopecia areata • Dyshidrotic hand eczema • Systemic lupus erythematosus	NBUVB NBUVB PUVA PUVA PUVA PUVA, Excimer UVA1 UVA1	
Summary of phototherapy's main clinical applications. NBUVB: Narrow band-UVB; PUVA: Psoralen plus UVA.		

Photopheresis

Mechanisms of action

Extracorporeal photochemotherapy, also known as extracorporeal photopheresis (ECP), is a promising form of immunomodulatory therapy. In apheresis, the blood of a donor or patient is passed through an apparatus that separates out one particular constituent and returns the remainder to the circulation. In photopheresis, a particular kind of apheresis, the majority of white blood cells are treated with 8-MOP, which becomes biologically active under exposure to UVA light, making pathogenic T cells, monocytes, natural killer cells, as well as B and T lymphocytes, susceptible to apoptosis. Furthermore, an immunological response follows the reinfusion of irradiated cells, with activation and differentiation of antigen-presenting cells (APCs, monocytes). APCs recognize apoptotic leukocytes through receptors that stimulate production of anti-inflammatory cytokines such as IL-10 and TGF- β . ECP also induces immune tolerance through the induction of Tregs, with increased production of IL-10 and TGF- β . ECP alters lymphocyte proliferation favoring a shift from a Th1 to a Th2 cytokine profile. Subcellular particles from apoptotic lymphocytes known as blebs show increased CD5 and CD8 and decreased CD28 and CD26

expression, surface markers consistent with an immunosuppressive phenotype. Apoptotic cells show increased expression of phosphatidylserine which is recognized by phagocytosing macrophages, thereby leading to their anti-inflammatory clearance [80].

Administration

ECP involves three stages – leucapheresis, photoactivation with a photosensitizer plus UVA and re-infusion of buffy coat – each treatment taking 3–4 h. One main difficulty of performing photopheresis results from the venous access. One cycle of ECP consists of two treatments on consecutive days, and one cycle is typically given every 2–4 weeks [81].

Safety

ECP's side effects are listed in Box 3 [80]. Side-effects are typically mild and transient and severe reactions such as vasovagal syncope or infections are uncommon. The lack of major side effects, including malignancies or infections, is very valuable in diseases for which alternative treatments are highly immunosuppressive [81]. Contraindications to ECP include severe renal, hepatic or cardiac impairment, coagulation disorders and hypersensitivity to psoralen compounds.

Indications

CTCL

Erythrodermic CTCL was the first disease to be treated with ECP in the early 1980s [82], an indication for which it received US FDA approval in 1988.

ECP is currently approved for the treatment of skin manifestations in all stages of CTCL, but there is good evidence to support the rejection of the use of ECP for the treatment of nonerythrodermic MF and fair evidence to support the use of ECP for erythrodermic MF, including stage III and IVA, and Sézary syndrome [80,83]. There are several guidelines on the use of ECP in patients with CTCL [83–85].

The role of ECP in treating earlier stages of CTCL may be limited. One plausible explanation are the low levels of circulating Sézary cells in the peripheral blood of these patients [80].

Other attempts to better characterize those CTCL patients who are more likely to respond to ECP revealed that patients with a ratio CD4:CD8 of <10 are more likely to respond than those with a ratio >10 and that a nonelevated LDH at the start of treatment tends to predict a better response to ECP compared with patients with an elevated LDH [15].

GVHD

Steroids and other immunosuppressive agents are the mainstay initial modes of therapy in the treatment of GVHD after allogeneic bone-marrow transplantation. ECP has been successfully used in refractory acute (aGVHD) and chronic (cGVHD) disease for the past 10 years [80,83,86].

Studies have found good results on cutaneous improvement of cGVHD but they were not as consistent in respect to liver and lung involvement [87,88]. Patients with progressive development of cGVHD may be more likely to respond to therapy versus those

Box 3. Side effects of extracorporeal photopheresis.

Common side effects

- Headache
- Nausea
- Chills
- Low-grade fever

Other side effects

- Hypotension
- Vasovagal syncope
- Septicemia
- Anemia
- Abnormal clotting response
- Skin infections over the venous access site
- Bacterial endocarditis

with *de novo* onset [89]. Another study reported a higher response rate in participants who started therapy earlier than 1 year after onset of cGVHD [88]. ECP may also work as a steroid-sparing treatment, more effective than conventional steroid-sparing immunosuppressants, as shown in a multicenter, prospective, Phase II clinical trial involving 95 patients with cutaneous cGVHD [90]. This may represent an advantage owing to its fairly safe profile [80].

ECP also seems promising in the treatment of patients with aGVHD, particularly those with cutaneous manifestations but also liver and gut involvement [80].

Solid organ transplants

In a randomized controlled trial, the addition of ECP to triple-drug immunosuppressive therapy significantly decreased the risk of cardiac rejection without increasing the incidence of infection [91]. Multiple case reports and other anecdotal data involving the use of ECP in transplant recipients of lung, liver, kidney and allogeneic stem cell transplantation can also be found in the literature [80]. ECP is effective for patients resistant to conventional treatment, particularly if started early [3]. Besides reversal of allograft rejection, a reduction in immunosuppressive therapy has also been frequently achieved [92,93].

Autoimmune diseases

Limited data has also been published regarding the use of ECP in a variety of autoimmune diseases [80].

A small number of patients with pemphigus vulgaris have been successfully treated with ECP, with tapering of concomitant immunosuppressive therapy. However, since clinical trials with a large cohort of patients have not yet been conducted, there is not sufficient evidence to support the use of ECP in patients with this disease [80].

Two randomized clinical trials found improvements in skin and joint severity scores in patients with systemic sclerosis after ECP [94,95], pointing to a favorable outcome when ECP is started early in the course of the disease, with a low incidence of side effects [94].

Box 4. Summary of the applications of extracorporeal photopheresis.**Main applications**

- Erythrodermic CTCL (IIIA, IV), Sézary syndrome
- Acute and chronic graft-versus-host disease
- Rejection in transplant recipients of heart, lung, liver, kidney and allogeneic stem cells

Other applications

- Autoimmune diseases: pemphigus vulgaris, systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis
- Atopic dermatitis
- Erosive lichen planus
- Crohn's disease
- Type 1 diabetes mellitus
- Prevention of restenosis in patients after percutaneous transluminal coronary angioplasty

CTCL: Cutaneous T-cell lymphoma.

An open-label pilot study published in 1992 stated an improvement of clinical skin and joint scores in seven of ten patients with systemic lupus erythematosus, although no significant changes were noted in laboratory parameters [96]. Since then only a few anecdotal cases have been reported in the literature [80].

Although at one time promising, the role of ECP for the treatment of rheumatoid arthritis is still questionable [80].

Other applications

Severe atopic dermatitis has been successfully treated with ECP, the first report going back to 1994 [97]. It has also been effective in the treatment of recalcitrant mucous and cutaneous erosive lichen planus [98,99]. Other recently reported potential applications of ECP might be refractory Crohn's disease, Type 1 diabetes mellitus and the prevention of restenosis in patients after percutaneous transluminal coronary angioplasty [80]. In Box 4, we summarize ECP's main clinical applications.

Key issues

- Narrow band-UVB (NB-UVB) currently represents the phototherapeutic modality of choice for the treatment of psoriasis.
- Psoralen plus UVA (PUVA) is an excellent treatment option for the early stages of cutaneous T-cell lymphoma (IA–IIA), here achieving long-lasting free-disease intervals. NB-UVB can also induce high rates of complete remission in early disease.
- NB-UVB is currently used more than PUVA in vitiligo because it has been shown to be at least as effective and possibly safer.
- UVA1 phototherapy is a highly effective monotherapy for acute, severe atopic dermatitis. NB-UVB and UVA/UVB phototherapy are effective treatments in chronic, mild and moderate disease.
- PUVA is a therapeutic option for extensive lichen planus and for lichenoid graft-versus-host disease.
- NB-UVB is currently the phototherapeutic option of choice in polymorphous light eruption. In solar urticaria, PUVA appears to be the most effective therapy available. Patients with other photodermatoses, including actinic prurigo, hydroa vacciniforme and erythropoietic porphyria, may benefit from NB-UVB or PUVA.
- Medium–high dose UVA1 therapy can provide significant benefit to patients with morphea, particularly linear and plaque subtypes of disease.
- Photopheresis is used in patients with erythrodermic cutaneous T-cell lymphoma, including stage III and IVA, and those with Sézary syndrome with good results.
- It has also been successfully used in refractory acute and chronic graft-versus-host disease, with cutaneous improvement. Similar benefit has also been reported in transplant recipients of heart, lung, liver, kidney and allogeneic stem cell transplantation.
- Limited data has been published regarding the use of extracorporeal photopheresis in a variety of autoimmune diseases, including pemphigus vulgaris, systemic sclerosis, systemic lupus erythematosus and rheumatoid arthritis.

Expert commentary

Phototherapy is an important treatment option for psoriasis and other skin diseases and is the first choice in case of contraindications for immunosuppression, such as patients with infections or cancer-prone patients. There are some discrepancies in the literature, regarding dosimetry recommendations, efficacy, risk of phototoxicity and long-term risks, particularly for NB-UVB and UVA-1 therapies. More data are needed in order to optimize these phototherapy modalities.

Besides advanced cutaneous T-cell lymphoma, theoretically, photopheresis is an exciting tool for the treatment of a variety of T-cell-mediated diseases and should be considered in severe and difficult-to-treat conditions, particularly in systemic sclerosis.

Five-year view

Phototherapy is a time-consuming treatment and this raises the question: what is the future position of phototherapy as a classical treatment for psoriasis in the era of biologics? The better understanding of phototherapy-induced molecular pathways and the identification of clinical and genetic predictors of treatment response will contribute to maintain the importance of phototherapy. On the other hand, it is attractive to speculate that at least an additive or possibly a synergistic effect can be expected in case of combination of phototherapy with a biologic. Targeted phototherapy using high-intensity UV devices may improve safety and efficacy, namely in difficult-to-treat cases of morphea, alopecia areata and vitiligo.

In the near future, more studies will corroborate the role of extracorporeal photopheresis in the prevention of solid organ transplant rejection and this therapeutic option will be available in more institutions worldwide.

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Aims

The major aims of this study are described below in two chapters, according to the model used. In chapter I, results were obtained in three models of skin cell cultures, monoculture of human melanotic melanoma cells (SK-mel-1), monoculture of human keratinocytes (HaCat) and co-culture of melanoma cells and keratinocytes and in chapter II, results were obtained from psoriasis patients submitted to UVB phototherapy.

Chapter I

- To evaluate the presence of a functional endocannabinoid system in the three models of skin cell cultures.
- To assess the role of cannabinoid receptors on melanogenesis in human melanotic melanoma cells alone or in co-culture with human keratinocytes (HaCat).
- To evaluate the influence of ultraviolet B (UVB) radiation on the expression of CB₁ and CB₂ receptor mRNA, endocannabinoid levels and endocannabinoid enzymes in the three models of skin cell cultures.
- To evaluate the influence of cannabinoid drugs in UVB-induced cell death of keratinocytes.
- To evaluate the role of catechol-*O*-methyltransferase (COMT) on melanogenesis and on UVB-induced cell death in human melanoma cells and human keratinocytes.
- To evaluate the influence of UVB irradiation on COMT activity in human melanoma cells and human keratinocytes.

Chapter II

- To evaluate erythrocyte catechol-*O*-methyltransferase activity in patients with psoriasis and to determine whether narrowband ultraviolet B (nbUVB) phototherapy modifies this activity.
- To assess the influence of nbUVB phototherapy in vitamin A and vitamin D serum levels in patients with psoriasis.
- To assess endocannabinoid plasma levels in psoriasis patients and to determine whether nbUVB phototherapy modifies these levels.
- To evaluate the impact of nbUVB phototherapy for psoriasis on clinical response, inflammatory and metabolic parameters and respective correlation with endocannabinoid plasma levels.

Chapter I

Ultraviolet B radiation on melanocyte and keratinocyte cell lines

*Inhibition of basal and ultraviolet B-induced melanogenesis
by cannabinoid CB₁ receptors: a keratinocyte-dependent effect*

Arch Dermatol Res 2011; 303(3):201-10

*Anandamine increases UVB - induced cell death of human
keratinocytes through transient receptor potential vanilloid-1 channel*

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Effect of ultraviolet B radiation on endocannabinoid metabolizing enzymes

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*Ultraviolet B radiation differentially modifies
catechol-O-methyltransferase activity in keratinocytes and melanoma cells*

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Inhibition of basal and ultraviolet B-induced melanogenesis by cannabinoid CB₁ receptors: a keratinocyte-dependent effect

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Abstract Ultraviolet radiation is the major environmental insult to the skin and stimulates the synthesis of melanin in melanocytes, which then distribute it to the neighboring keratinocytes where it confers photo-protection. Skin color results from the paracrine interaction between these two cell types. Recent studies suggest that endocannabinoids are potential mediators in the skin. Here, we investigated whether cannabinoid drugs play a role in melanogenesis and if ultraviolet radiation modifies the cutaneous endocannabinoid system. We used human melanotic melanoma cell line (SK-mel-1) in monoculture or co-culture with human keratinocytes (HaCat). Endocannabinoid levels, cannabinoid receptors expression, and melanin content were evaluated under basal conditions and after ultraviolet-B irradiation (311 nm). We provide evidence that human melanoma cells (SK-mel-1) express CB₁ receptors, and when in co-culture with keratinocytes (HaCat), the selective CB₁ receptor agonist arachidonyl-2-chloroethylamide

(ACEA 1 and 10 μM) inhibited (by 33.4 and 37.3%, respectively) basal melanogenesis. In addition, ultraviolet-B-induced melanogenesis in co-cultures was abolished by ACEA 10 μM. Both ACEA inhibitory effects were reversed by AM251 (1 μM), a selective CB₁ antagonist. Furthermore, ultraviolet-B radiation increased endocannabinoids levels only in keratinocytes, whereas CB₁ cannabinoid receptor expression was up-regulated only in melanoma cells. Our results collectively suggest that ultraviolet radiation activates paracrine CB₁-mediated endocannabinoid signaling to negatively regulate melanin synthesis. The endocannabinoid system in the skin may be a possible target for future therapies in pigmentary disorders.

Keywords Melanin · Melanoma · Ultraviolet radiation · Cannabinoids · Co-culture

Introduction

In the last two decades, research has dramatically increased the knowledge of endocannabinoid biology and pharmacology. The cloning of two cannabinoid receptors, CB₁ and CB₂ and the discovery of their endogenous ligands, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), increased the interest in understanding the physiological role of the endocannabinoid system. This research provided evidence that there is a wide range of disorders in which endocannabinoid levels and cannabinoid receptor density are increased and that this up-regulation can either counteract or contribute to the pathological mechanisms reviewed by Di Marzo and Pertwee [4, 14].

The skin functions as a neuro-endocrine organ with functional epidermal units formed by one melanocyte surrounded by keratinocytes in intimate paracrine

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regulation. The distribution and expression of CB₁ receptors were uniformly found in skin biopsies taken from different body sites [20] and recent studies strongly suggest the existence of a functional cutaneous endocannabinoid system reviewed by Biro et al. [2]. There is evidence that human keratinocytes have the biochemical machinery to bind, synthesize and hydrolyze endocannabinoids [9] and, in a CB₁ receptor-dependent manner, cannabinoids regulate keratinocyte differentiation [12]. More recently, a protective role of the endocannabinoid system in contact allergic dermatitis was described [7]. A previous study carried out in mouse epidermal JB6 P(+) cells showed that UVB irradiation can increase the levels of AEA and PEA [1]. In addition, it was demonstrated that human and mouse melanoma cells contain cannabinoid CB₁ and CB₂ receptors [3]. As activation of these receptors increases apoptosis in melanoma cells, they might represent novel targets for the treatment of melanoma. Very recent data on CB₁ and CB₂ knockout mice suggest that cannabinoid receptors are required for ultraviolet-induced inflammation and skin cancer development [22]. Melanocytes play a fundamental role in ultraviolet (UV) protection by synthesizing the pigment melanin in discrete organelles, the melanosomes. Once filled with melanin, melanosomes are transferred to keratinocytes, where they form a supranuclear cap to protect DNA against UV irradiation reviewed by Schallreuter [18]. The lack of safe and efficient drugs for skin pigment regulation or protection of melanocytes against UV light increases the interest in studying the cutaneous endocannabinoid system and its response to UV. However, direct evidence for the presence of a functional endocannabinoid system in melanocytes and data on the pharmacological effects of cannabinoids on melanin synthesis are lacking in the literature. Although UV radiation is the major environmental insult to the skin, and the endocannabinoid system is suggested to play a pro-homeostatic role, the influence of UV radiation on cutaneous endocannabinoid system activity is unknown. On the other hand, due to their highly lipophilic nature, the potential use of cannabinoid drugs for skin diseases is an interesting field of research, because they can be used as topical preparations without important side effects.

In order to investigate the response of the cutaneous endocannabinoid system to UV radiation and the proposed role of cannabinoid drugs in the regulation of melanogenesis, we chose two models of cell cultures, one with human melanotic melanoma cells alone and the other in co-culture with keratinocytes. Specifically, we evaluated cell melanin content under basal conditions and after acute low dose UVB irradiation in the absence and in the presence of cannabinoid drugs. Furthermore, we measured the expression of cannabinoid receptors (CB₁ and CB₂) and endocannabinoids levels (AEA, 2-AG) in melanoma cells and

keratinocytes under basal conditions and after UVB acute irradiation. The effect in cell viability was also quantified.

Materials and methods

Materials

Minimum essential medium Eagle (MEM), Dulbecco's minimal Eagle's medium (DMEM), antibiotic antimycotic solution, HEPES, synthetic melanin, and human anti-cannabinoid receptor 1 antibody were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Rabbit anti-cannabinoid receptors CB₁ and CB₂ antibodies were purchased from Chemicon® International, Inc. (Temecula, CA, USA). Fetal bovine serum (FBS) and trypsin were purchased from Gibco (Paisley, UK). NaHCO₃ and EDTA were purchased from Merck (Germany). Arachidonoyl-2'-chloroethylamide (ACEA) was purchased from Biotrend Chemicals AG (Zurich, Switzerland) and AM251 from Tocris Bioscience (Bristol, UK).

Cell lines and culture conditions

Human SK-mel-1 melanoma cells (ATCC no. HTB-67) were grown routinely in MEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, 25 mM HEPES, and 0.26 M NaHCO₃, at 37°C in a 5% CO₂ (v/v), humidified atmosphere.

Human HaCaT keratinocytes, provided by Prof. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany), were grown in DMEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, and 0.44 M NaHCO₃. Cells were maintained at <80% confluency, at 37°C in a 5% CO₂ (v/v), humidified atmosphere.

SK-mel-1 and HaCaT cells ratio 1.8:2, as described in [16] were co-cultivated in six-well tissue culture plates, 96 h prior to the beginning of the experiment, in DMEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, and 0.44 M NaHCO₃. However, due to their different proliferation rates, the ratio of melanocytes:keratinocytes changes in the first 48 h, eventually reaching a stable ratio of 1:4 (which is the one used during the experiments). Co-cultures were incubated 2 h before the first UVB irradiation with DMEM medium containing either ACEA 1 µM, ACEA 10 µM, AM251 1 µM, AM251 1 µM + ACEA 1 µM or AM251 1 µM + ACEA 10 µM and the medium containing the different drugs tested was replaced every 24 h, until the end of the experiment. Cannabinoid agonists and antagonists were directly applied at a final ethanol concentration <0.01% (v/v). No significant influence of the vehicle was observed on any of the parameters determined.

Western blot

Western blot analysis was carried out as previously described [10]. Briefly, the membranes were incubated with either rabbit anti-cannabinoid receptor 1 antibody (diluted 1:250) or rabbit anti-cannabinoid receptor 2 antibody (diluted 1:500) for 2 h and then were incubated with the fluorescently labeled secondary antibody (goat anti-rabbit from Rockland). The specificity of the anti-cannabinoid receptor antibodies was assessed by antigen preabsorption with the corresponding blocking peptides. Membranes were imaged by scanning at 700 nm with the Odyssey Infrared System (LICOR Biosciences, Lincoln, NE, USA).

UVB irradiation

Cells in co-culture were washed with phosphate-buffered saline (PBS) and then exposed to low (3 mJ/cm²) and high (7 mJ/cm²) dose of UVB light wavelength (312 nm), emitted by a pre-heated, filtered, UV lamp (VL-6.M, Vilber Lourmat, France) at $t = 0$ min, $t = 24$ and 48 h. This was followed by 48-h period of incubation after which the cells were harvested [10 min incubation with EDTA (0.05%, pH 7.4) and 5 min incubation with trypsin] for analysis. SK-mel-1 cells in monoculture were washed with PBS, resuspended in 1 ml of PBS and returned to the plate well for exposure to low (3 mJ/cm²) and high (7 mJ/cm²) dose UVB light under the same conditions as the cells in co-culture. Following the 48-h period of incubation, these cells did not require incubation with EDTA and trypsin for harvesting, as they grow in suspension. HaCat cells in monoculture were washed with PBS and then exposed to low (30 mJ/cm²) and high (60 mJ/cm²) dose UVB light at $t = 0$ min. The low and high irradiation doses were defined according to cell type based on the UVB radiation effect on cell viability. With low UVB doses, cell viability was unchanged; by contrast the high doses produced an equivalent toxic effect (around 40% loss).

Non-irradiated controls were not exposed to UVB light, but subjected to all other manipulations. UV dosimetry was performed with a Waldmann 585.100 UV-meter (Herbert Waldmann GmbH & Co., Villingen-Schwenningen, Germany).

Melanin and cell viability determination

Harvested cells were centrifuged at 300×*g* for 5 min at room temperature, washed twice with PBS and resuspended in 1 ml of PBS. From this suspension, 180 µl were transferred into a black/clear bottom MicrotestTM 96-well Assay Plate, OpliluxTM (BD Biosciences, Franklin Lakes, NJ, USA) for cell viability assay. Cell viability was

measured using calcein-AM (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions. Each well containing 180 µl of cell suspension was loaded with 20 µl of calcein-AM (10 µM, in PBS solution). Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths using a SpectraMax, Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at 40-min incubation at room temperature.

The melanin content of cells was determined according to [17]. Briefly, cells were centrifuged at 300×*g* for 5 min, resuspended in 500 µl of a NaOH (1 M, in 10% DMSO) solution and incubated at 80°C for 2 h. This was followed by centrifugation at 500×*g* for 10 min, and the resulting supernatant was used for measurement of melanin at 420 nm in a PowerWaveTM HT microplate scanning spectrophotometer (BioTek Instruments, Inc., VT, USA). Synthetic melanin was used to construct a standard curve for the range of 1–100 µg/ml. Melanin absorbance was measured at 420 nm wavelength.

Real-time polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction (RT-PCR) was carried out as previously described [6]. Total RNA from keratinocytes and melanoma cells in co-cultures ($n = 5$, per group) was isolated using the PureZOL RNA isolation reagent (Bio-Rad, CA, USA). Total RNA (1 µg per sample) was DNase treated and reverse transcribed according to the manufacturer's instructions (iScriptTM cDNA Synthesis Kit, Bio-Rad). For RT-PCR, 50 µl of amplification mixture (iQTM SYBR[®] Green Supermix, Bio-Rad) was used containing 20 ng of reverse transcribed RNA and specific primers (250 nM) (Sigma) for *CNR1* (sense: 5'-TG ACATTCAGTACGAAGACATCAA-3'; anti-sense: 5'-CA CTCTATGTCCATGAAGTTCTCC-3'), *CNR2* (sense: 5'-TG GGAGAGGACAGAAAACAAC-3'; anti-sense: 5'-GAG CTTGTCTAGAAGGCTTTGGG-3') and *GAPDH* (sense: 5'-AGCCACATCGCTCAGACAC-3'; anti-sense: 5'-GCC CAATACGACCAAATCC-3'). Reactions were run in duplicates (20 µl) on a MJ Mini detector (Bio-Rad, CA, USA). The cycling conditions were 15 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s, at 58°C for 30 s, and at 72°C for 30 s. Results were normalized to *GAPDH* values and expressed as log₂ (sample/control).

Extraction, purification, and quantification of endocannabinoids and PEA

Cells were homogenized in chloroform:methanol:Tris-HCl 50 mM (2:1:1) containing 10 pmol of d8-AEA, d4-palmitoylethanolamide (PEA) and d5-2-arachidonoylglycerol (2-AG) as internal standards. Homogenates were centrifuged at

13,000×*g* for 16 min (4°C), the aqueous phase plus debris were collected and extracted again twice with 1 vol. of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized extracts were resuspended in chloroform:methanol (99:1, v/v). The solutions were then purified by open bed chromatography on silica as previously described [15]. Fractions eluted with chloroform:methanol (9:1, v/v) and containing AEA, PEA, and 2-AG were collected, the excess solvent evaporated with a rotating evaporator, and extracts analyzed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (APCI-LC-MS) carried out under conditions described previously [15] and allowing the separations of 2-AG, PEA and AEA. Results are expressed as pmol/mg of lipid extract.

Statistical analysis

Data are presented as mean ± SD. Student's *t* test was used to analyze differences. Values of *P* < 0.05 are considered significant.

Results

Expression of cannabinoid receptors

Cannabinoid CB₁ and CB₂ receptors mRNA expression was identified by quantitative real-time PCR using RNA extracted from SK-mel-1 and HaCat cells. Cannabinoid CB₁ and CB₂ receptors protein expression was identified by western blot using SK-mel-1 cell extracts (Fig. 1).

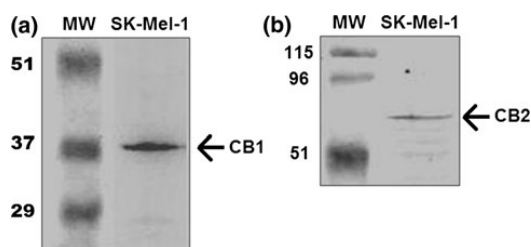


Fig. 1 Western blot analysis of cannabinoid CB₁ and CB₂ receptors in SK-mel-1 cell extracts. Molecular mass markers (*MW*) are shown on the left in kDa. A single band of the expected size (37 kDa for CB₁ and 60 kDa for CB₂) was revealed following incubation with the respective anti-cannabinoid receptor antibody. The specificities of the anti-cannabinoid receptor antibodies were assessed by antigen preabsorption with the corresponding blocking peptides (not shown)

Time-course of basal melanogenesis

In non-irradiated control situation, basal melanin production increased in a time-dependent manner, both in co-cultures of human melanotic melanoma cells (SK-mel-1) with human immortalized keratinocytes HaCat (SK-mel-1-HaCat) and monocultures of human melanoma cells (SK-mel-1) alone. However, melanin content was significantly increased in co-culture model when compared to melanoma cells alone. After 48-h incubation, this difference was already evident, reaching a 2.5-fold increase at 96 h (Fig. 2).

Effect of CB₁ receptor activation on basal melanogenesis

In co-culture model, time-course experiments showed that the CB₁ selective agonist ACEA 1 μM, had an inhibitory effect on melanogenesis. Although this inhibitory effect was already observed after 72 h, it was more evident (37.3% reduction) after 96 h (Fig. 2). By contrast, until 96-h incubation time, ACEA 1 μM did not change melanin content in melanoma cells alone (data not shown).

Since the 96-h time point was the one at which we found more significant differences, we chose this incubation time to evaluate the effect of all tested compounds.

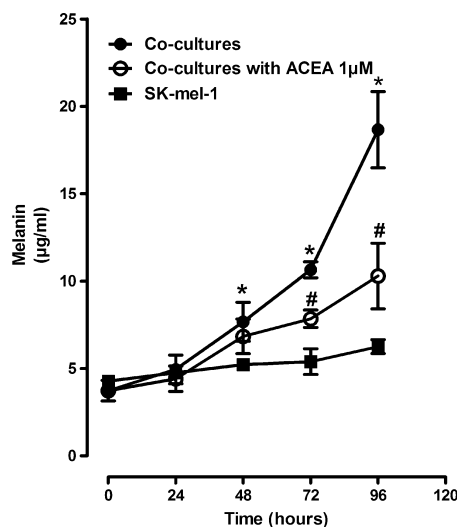


Fig. 2 Time-course of basal melanogenesis. Basal melanogenesis in monoculture of SK-mel-1 (filled square), in co-culture of keratinocytes (HaCat) with melanocytes (SK-mel-1) under control situation (filled circle) and in the presence of ACEA 1 μM (open circle). Values are mean ± SD (*n* = 6) **P* < 0.05 compared to monocultures, #*P* < 0.05 compared to co-cultures under control conditions

In the co-culture model, both 1 and 10 μM of ACEA had a similar inhibitory effect in melanogenesis (37.3 and 33.4%, respectively), however, in melanoma cells alone, even the higher concentration tested (10 μM) did not change melanin content (Fig. 3a).

The specific CB₁ antagonist AM-251 (1 μM), which alone did not modify melanin content, completely reverted the melanogenesis-inhibitory effect of both 1 and 10 μM of ACEA in the co-culture (Fig. 3a).

Two well-known melanogenesis inhibitors, kojic acid and hydroquinone, were used as positive controls. The ACEA inhibitory effect on melanin content had the same magnitude as kojic acid (1 mg/ml) in co-cultures.

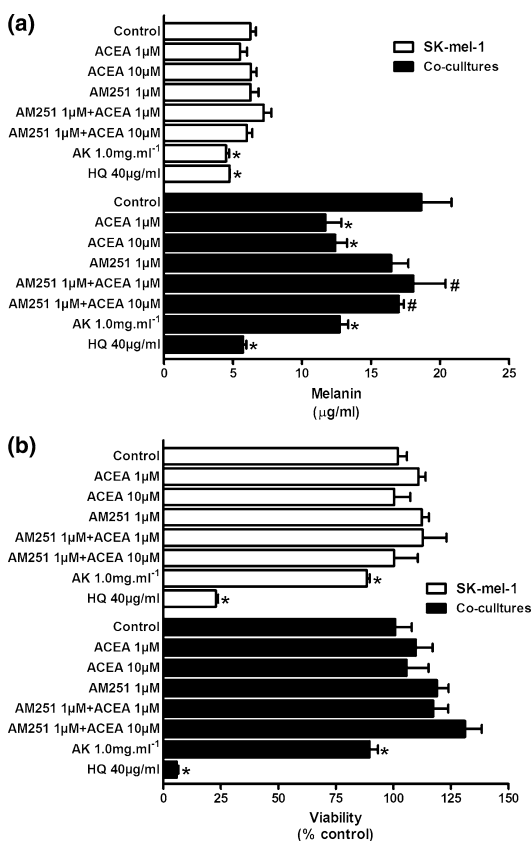


Fig. 3 Melanin content and cell viability in monocultures (SK-mel-1) and co-cultures (SK-mel-1-Ha-Cat). **a** Cellular melanin content in monocultures (SK-mel-1) and co-cultures (SK-mel-1-Ha-Cat), after 96-h incubation. Melanin content was determined in total cell extracts and expressed in $\mu\text{g/ml}$. The results were normalized to respective cell viability. **b** Cell viability in monocultures (SK-mel-1) and co-cultures (SK-mel-1-Ha-Cat), after 96 h incubation. Cell viability was expressed as the percentage of untreated controls, set to 100. Bars represent mean \pm SD ($n = 10$) * $P < 0.05$ compared to respective control values, # $P < 0.05$ compared to respective ACEA values

Interestingly, unlike ACEA, kojic acid inhibited melanogenesis also in melanoma cells monocultures (Fig. 3a).

On the other hand, both kojic acid and hydroquinone had a significant cytotoxic effect while ACEA (1 and 10 μM) did not change cell viability (Fig. 3b).

Effect of UVB irradiation on melanogenesis

Initially, we investigated the effect of different UVB irradiation doses (3–24 mJ/cm^2) on melanogenesis in co-cultures and melanoma cells alone (data not shown). We found that, in the co-culture model, three consecutive, daily UVB exposures at 3 mJ/cm^2 significantly increased cellular melanin content ($\sim 40\%$ over the controls) (Fig. 4a). Interestingly, the same UVB treatment did not significantly

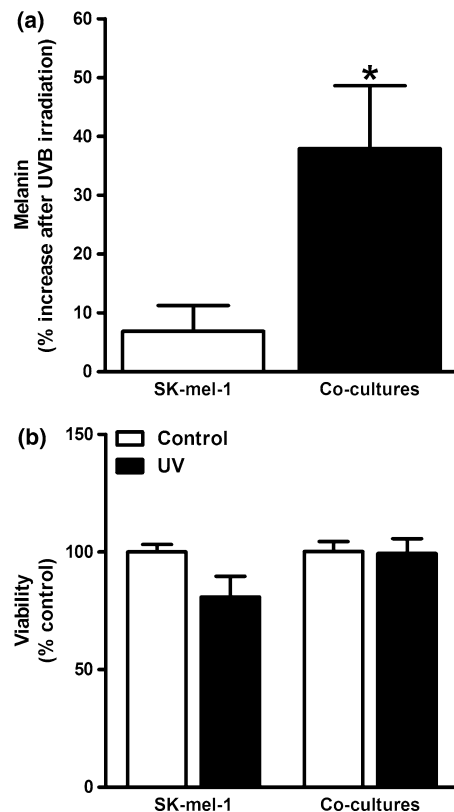


Fig. 4 Effect of UVB irradiation on melanin content and cell viability. **a** Effect of UVB irradiation (3 mJ/cm^2) on melanogenesis in monocultures (SK-mel-1) and in co-cultures (SK-mel-1-HaCat). Results were expressed as percentage of increase in melanin content after irradiation, over the non-irradiated controls. **b** Effect of UVB irradiation (3 mJ/cm^2) on cell viability in monocultures (SK-mel-1) and in co-cultures (SK-mel-1-HaCat). Cell viability results were expressed as the percentage of untreated controls set to 100. Bars represent mean \pm SD ($n = 15$) * $P < 0.05$ compared to monocultures

modify melanin content of melanoma cells alone (Fig. 4a). On the other hand, this UVB dose (3 mJ/cm^2) did not lead to a significant decrease in cell viability (Fig. 4b). UVB irradiation with doses $>3 \text{ mJ/cm}^2$ had a dose-dependent cytotoxic effect in SK-mel-1 monocultures (data not shown).

Effect of CB₁ receptor activation on UVB-induced melanogenesis

We also evaluated whether stimulation of CB₁ receptors could modulate the increase in melanin content observed in co-culture model after UVB irradiation. Therefore, we performed UVB experiments after 96-h incubation with the CB₁ receptor-selective agonist ACEA (1 and $10 \mu\text{M}$) and with the CB₁ receptor selective antagonist AM-251 ($1 \mu\text{M}$) (Fig. 5). We found that ACEA $10 \mu\text{M}$ abolished the increase in melanin content observed after UVB irradiation. Furthermore, AM-251, which alone did not modify UVB-induced melanogenesis, reversed the inhibitory effect of ACEA $10 \mu\text{M}$. Again, the melanogenesis inhibitor, kojic acid was used as a positive control but, comparatively, ACEA $10 \mu\text{M}$ exhibited a stronger effect on UVB-induced melanogenesis (Fig. 5).

Effect of UVB irradiation on cannabinoid receptor mRNA expression

CB₁ and CB₂ cannabinoid receptors mRNA expression was identified by quantitative real-time PCR using RNA extracted from SK-mel-1 and HaCat cells from co-cultures

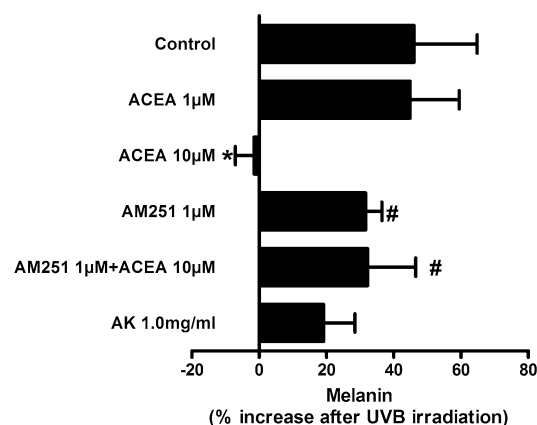


Fig. 5 Effect of cannabinoids on UVB-induced melanogenesis in co-cultures (SK-mel-1-HaCat). Results were expressed as percentage of variation in melanin content after irradiation, over the non-irradiated controls. Bars represent mean \pm SD ($n = 18$) * $P < 0.05$ compared to control values, # $P < 0.05$ compared to respective ACEA $10 \mu\text{M}$ values

under basal conditions and after UVB irradiation. We found that, in melanoma cells, acute UVB irradiation (3 mJ/cm^2) significantly up-regulated CB₁ receptor mRNA (Fig. 6a), whereas in keratinocytes neither CB₁ nor CB₂ mRNA expression was modified by UVB (Fig. 6b, d).

Effect of UVB irradiation on endocannabinoids levels

We quantified the levels of the two most studied endocannabinoids, AEA and 2-AG, and of palmitoylethanolamide (PEA), in SK-mel-1 monocultures, in HaCat monocultures and in co-cultures (SK-mel-1, HaCat) under basal conditions and after acute low and high dose UVB irradiation. Our results, shown in Fig. 7, indicate that AEA and PEA were elevated in HaCat cells irradiated with low UVB dose (1.59 ± 1.09 and $16.64 \pm 3.78 \text{ pmol/mg}$ of lipid extract) when compared with non-irradiated control cells (0.15 ± 0.08 ; $6.37 \pm 0.59 \text{ pmol/mg}$ of lipid extract). The levels of 2-AG were even more strongly elevated in irradiated HaCat cells ($13.95 \pm 1.91 \text{ pmol/mg}$ of lipid extract) when compared with non-irradiated controls ($2.87 \pm 1.18 \text{ pmol/mg}$ of lipid extract). By contrast, although basal levels of AEA and 2-AG were higher in melanoma cells (3.49 ± 2.09 and $14.32 \pm 1.22 \text{ pmol/mg}$ of lipid extract) than in keratinocytes (0.15 ± 0.08 and $2.87 \pm 1.18 \text{ pmol/mg}$ of lipid extract), they remained unaltered by UVB irradiation. In co-cultures, the ratio 2-AG:AEA is 0.6 under baseline conditions and increases to 8.7 and 8.1 after low and high dose UVB irradiation, respectively. In contrast to AEA, both 2-AG and PEA levels were significantly increased by UVB irradiation. In general, the levels of 2-AG and AEA after irradiation were lower in co-cultures than in melanoma cell line monocultures, whereas the levels of PEA were significantly higher (Fig. 7).

Discussion

In the present study, we provide the first evidence that cannabinoid CB₁ receptors exert an inhibitory effect on melanin production both under basal conditions and after UVB irradiation. Furthermore, we show that the endocannabinoids AEA and 2-AG, and PEA, a non-cannabinoid receptor active congener of AEA with anti-inflammatory actions [15], are produced by human melanoma cells at physiologically relevant concentrations. We demonstrated that UVB increases the mRNA expression of CB₁ receptors in melanoma cells but not in keratinocytes, and that keratinocytes respond to UVB radiation with an elevation of 2-AG and AEA levels not observed in melanoma cells. Taken together, our data support the concept that human melanoma cells are both sources and, particularly, targets

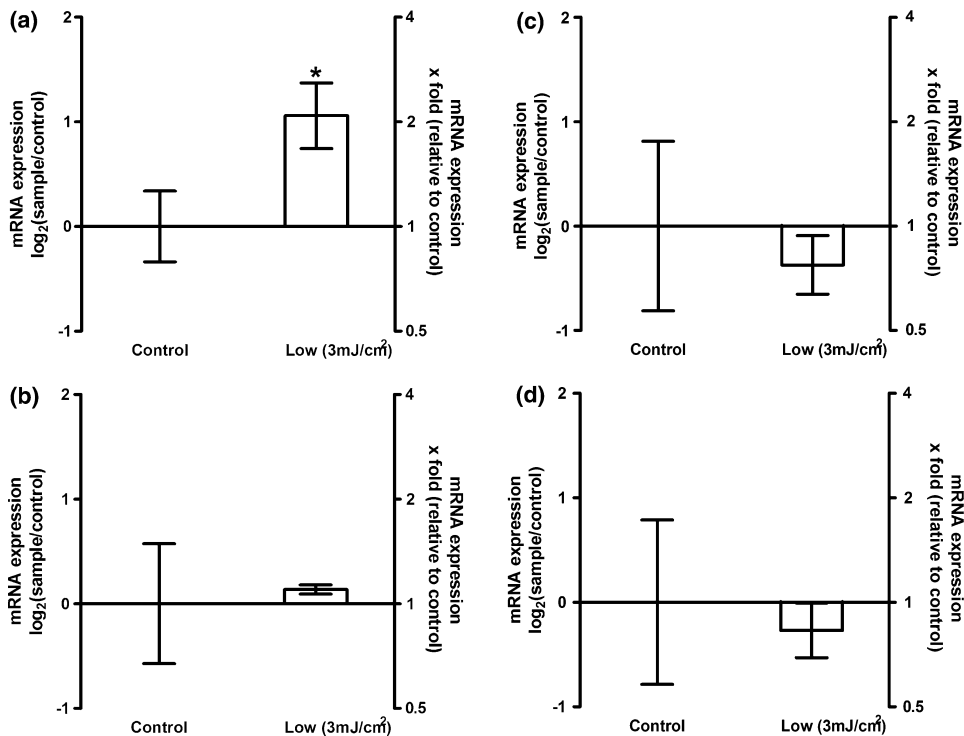


Fig. 6 Effect of UVB irradiation on cannabinoid receptors mRNA expression. Cannabinoid CB₁ receptor mRNA expression before (Control) and after 3 mJ/cm² UVB irradiation (Low) in melanocytes (SK-mel-1) (a) and in keratinocytes (HaCat) (b). Cannabinoid CB₂

receptor mRNA expression before (Control) and after 3 mJ/cm² UVB irradiation (Low) in melanocytes (SK-mel-1) (c) and in keratinocytes (HaCat) (d). Bars represent mean ± SD (n = 5). *P < 0.05 compared to respective non-irradiated controls

of endocannabinoids, which function as negative regulators of melanin synthesis after UVB radiation. Our data suggest that endocannabinoids produced by keratinocytes after UVB radiation might act at melanocyte CB₁ receptors in a paracrine way, to counteract the excessive melanin production.

Although, melanoma cell lines have been extensively used to study melanogenesis [17, 21] this may constitute a limitation when extrapolating our results to normal skin physiology. Several co-culture models with keratinocytes and melanocytes have been developed, namely with HaCat cells [19], furthermore, a co-culture identical to the one used here (SK-mel-1 and HaCat cells) was previously described as a suitable in vitro model to study the transfer of melanin/melanosomes from melanocytes to keratinocytes [16]. The present co-culture model evaluates melanogenesis without the proliferative capacity and donor phototype variability limitations of normal human melanocytes. In fact, melanoma cells co-cultured with keratinocytes are a better model to study the melanogenic response and the cannabinoid influence on the melanogenic

response is only observed in the co-culture model. In the “epidermal melanin unit”, the close contact between melanocytes and keratinocytes allows not only the transfer of newly synthesized melanin into keratinocytes, but also an intensive cross-talk between the two cell types. The observed inhibitory effect of CB₁ on melanogenesis in co-cultures but not in monocultures underlines the important symbiotic relationship between melanocytes and keratinocytes. Since both cells express CB₁ receptors, it can be speculated that cannabinoids acting on these receptors in keratinocytes inhibit the release of keratinocyte-derived paracrine melanogenic mediators. Alternatively, it is possible that CB₁ receptors directly act on melanoma cells to inhibit melanogenesis only when the conditions to produce melanin are optimized by the presence of keratinocytes.

To investigate whether there is a difference in the melanogenic response to UVB irradiation of melanoma cells in monoculture as compared to those co-cultured with keratinocytes, we exposed both types of culture to UVB irradiation. Unlike co-cultures, which exhibited a melanogenic response to UVB irradiation (3 mJ/cm²), melanoma

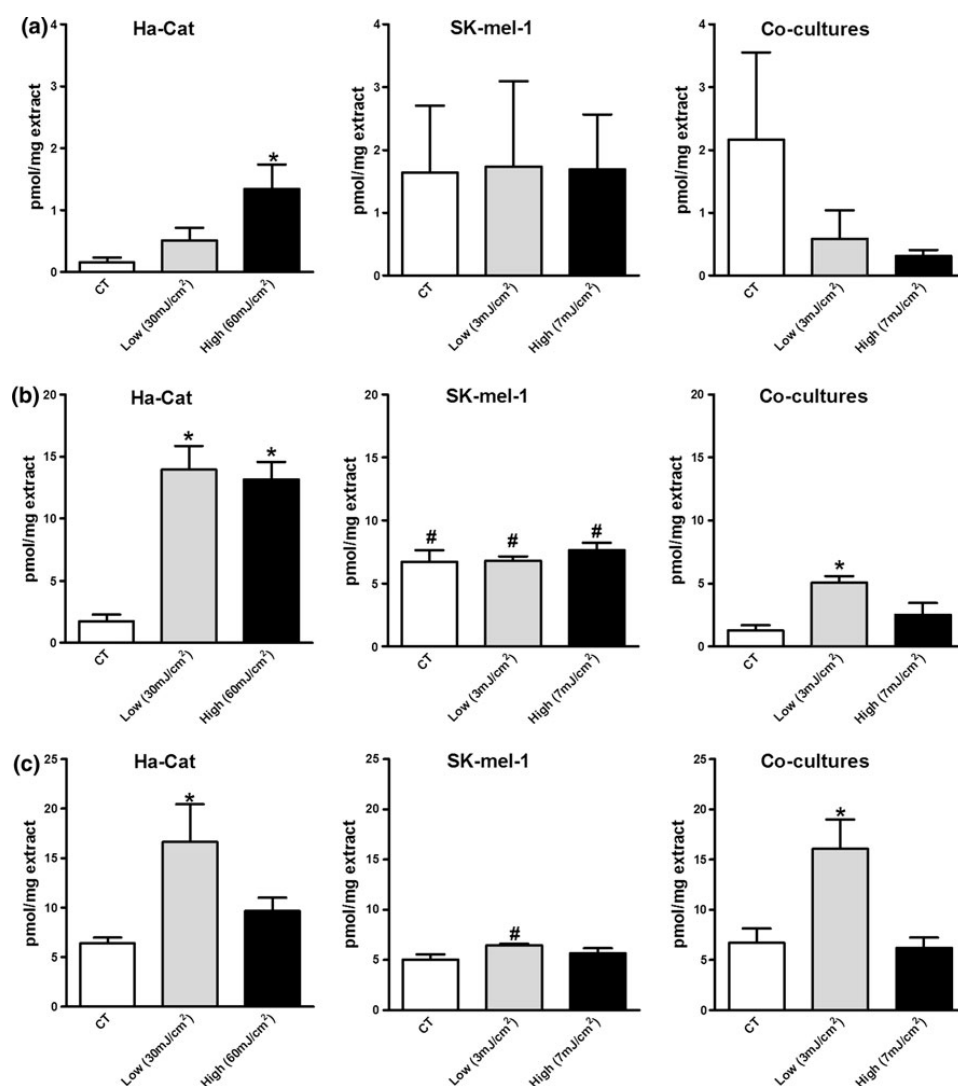


Fig. 7 Effect of UVB irradiation on endocannabinoids and PEA levels. Anandamide (a), 2-AG (b) and PEA (c) levels in HaCat monocultures, SK-mel-1 monocultures and in co-cultures (HaCat-SK-mel-1), either non-irradiated (CT) or after irradiation with UVB at

low and high doses. Data are mean \pm SD ($n = 4$ separate determinations). * $P < 0.05$ compared to respective non-irradiated controls # $P < 0.05$ compared to respective experimental condition in co-cultures

cells alone did not show any increase in melanin content after irradiation with the same dose. It is known that UVB irradiation of keratinocytes triggers the release of cytokines, growth factors, and other mediators such as endothelin-1, nitric oxide or proopiomelanocortin-derived peptides known to be effective modulators of melanin synthesis in a paracrine way [5, 13]. This may explain the differences observed between mono- and co-cultures in their response to UVB. Under basal co-culture conditions,

the CB₁-mediated inhibitory effect was also observed on UVB-induced melanogenesis, and was comparable to the effect of the well-known hypo-pigmentary agent, kojic acid. From these results, we suggest that keratinocytes play a major role in cannabinoid-mediated inhibition of basal and, particularly, UVB-induced pigmentation.

Endocannabinoid levels were stimulated by UVB irradiation in keratinocytes and co-cultures but not in melanoma cells alone. Interestingly, the co-cultures contain

overall less endocannabinoids (except PEA) than melanoma cells alone under basal conditions, and even more following UVB irradiation. This phenomenon may be due to the presence of endocannabinoid-degrading enzymes in keratinocytes [9] in levels possibly up-regulated by UVB. This could suggest a new endocannabinoid-mediated mechanism tightly regulating UVB-induced melanogenesis.

Besides melanogenesis, acute UVB irradiation also induces skin inflammation and immunosuppression. The standard model for studying UVB-induced immunosuppression is the UVB-ability to block either induction or elicitation of cutaneous allergic sensitization. It is well known that UVB irradiation of keratinocytes induces the synthesis and secretion of a whole set of pro-inflammatory and immunosuppressive soluble factors. Interestingly, a recent study [7] demonstrated that endocannabinoids attenuate the cutaneous allergic response and, more recently, a protective role of PEA in the same context was described [15]. Also antagonism of CB1 cannabinoid receptor in human HaCat keratinocytes was associated with the increased expression of pro-allergic chemokines [8]. Since we found here that UVB irradiation is accompanied by up-regulation of both 2-AG and PEA levels, we hypothesize that, whilst cutaneous endocannabinoids may play a role in both UVB-induced melanogenesis and immunosuppression, PEA might represent an endogenous protective agent against UVB-induced inflammation. These phenomena might contribute to the well-known therapeutic effect of UVB radiation in chronic inflammatory skin diseases. Endocannabinoid signals involved in skin inflammation are complex and remain poorly understood. Overall, anandamide appears to mediate an anti-inflammatory effect since inhibition of its degradation significantly reduced inflammation [7] and there is growing evidence suggesting that 2-AG modulates the inflammatory response by acting on the cannabinoid CB₂ receptor [11]. Interestingly, we found a significant increase in 2-AG:AEA ratio after UVB irradiation in co-cultures, suggesting that endocannabinoids, namely 2-AG, may play a role in UVB-induced inflammation.

A recent study by Zheng et al. [22] suggested that CB receptors and the related signaling pathways might be involved in the promotion of *in vivo* skin carcinogenesis. In CB₁ and CB₂ double gene-deficient mice, there was a marked decrease in UVB-induced skin carcinogenesis. This finding was quite surprising in view of previous data indicating that the endocannabinoid system is protective against the growth and spreading of skin cancer and melanoma [3]. Since the major known function of melanin is to provide protection against ultraviolet-induced DNA damage by absorbing and scattering UV radiation, our *in vitro* results provide a possible explanation to the findings of Zheng and colleagues.

In conclusion, the present study suggests that ultraviolet radiation activates paracrine CB₁-mediated endocannabinoid signaling to negatively regulate melanin synthesis. Furthermore, endocannabinoids might act as hypopigmentary agents either under basal conditions or after ultraviolet exposure. Although the use of cannabinoids in medicine is limited by their psychotropic effects, in cutaneous diseases these effects could be avoided by their topical use on the skin.

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Anandamide increases UVB-induced cell death of human keratinocytes through transient receptor potential vanilloid-1 channel

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Abstract

Endocannabinoids like anandamide (AEA) play a key role in skin biology. Exposure to high-dose ultraviolet B (UVB) radiation induces keratinocyte apoptosis and increases the synthesis of anandamide (AEA) in keratinocytes. Published data on the effects of cannabinoids on the regulation of keratinocyte cell death are conflicting. Moreover the influence of cannabinoids on UVB-induced cell death has never been studied. Here, we investigated whether AEA and other cannabinoid receptors ligands modify keratinocyte survival and UVB-induced keratinocyte death. Cell viability in human immortalized HaCat keratinocytes was evaluated under basal conditions and 48 h after high-dose (70 mJ/cm²) UVB (311 nm) irradiation. Pretreatment of HaCat cells with AEA at high concentrations (30 μ M) significantly increased UVB-induced cell death. This effect was replicated by the transient receptor potential vanilloid-1 channel (TRPV1) agonist capsaicin (10 μ M). The selective CB₁ antagonist AM-251 (1 μ M) did not modify the effect of AEA. In contrast, the selective TRPV1 antagonist, capsazepine (5 μ M), which alone did not modify cell viability, abolished the increase in UVB-induced cell death of either AEA or capsaicin. Our results suggest that AEA potentiates cell death induced by UVB radiation through TRPV1 in human keratinocytes. Endocannabinoids participate in the adaptive skin response to UVB radiation and could be useful in combination to phototherapy for treatment of hyperproliferative dermatoses such as psoriasis.

Introduction

The endocannabinoid system is not limited to the central nervous system [4] [9] and has a role in skin physiology [1]. Several human skin cells produce the major endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and express the enzymes responsible for their synthesis and metabolism [8,11,7].

Endocannabinoids bind to G-protein-coupled cannabinoid receptors, CB₁ and CB₂ [10]. AEA but not 2-AG is also an agonist of the transient receptor potential vanilloid-1 channel (TRPV1) [10]. TRPV1, CB₁ and CB₂ receptors were identified, both *in situ* and *in vitro*, on different cutaneous cells, including human keratinocytes [8] [7] [12]. Cannabinoids may induce apoptosis in different cell types and they have been tested as potential antitumoral agents. However, published data on the effects of cannabinoids on the regulation of keratinocytes cell death are conflicting. Wilkinson *et al* [16] found that Δ^9 -tetrahydrocannabinol as well as synthetic cannabinoid agonists inhibited growth of cultured transformed human epidermal keratinocytes, yet these effects were CB₁ and CB₂ independent. In another study, activation of both types of cannabinoid receptors by synthetic agonists induced the apoptotic death of tumorigenic epidermal cells whereas the viability of non-

tumorigenic human (HaCat cells) and murine (MCA3D) keratinocytes remained unaffected [3]. In addition, it was observed that metabolism of AEA by cyclooxygenase-2 (COX-2) is important for AEA-induced cell death in tumorigenic keratinocytes and that neither CB₁ nor CB₂ nor TRPV1 receptors were required for this response [13] [5]. We have recently reported that the cutaneous endocannabinoid system responds to ultraviolet B (UVB) irradiation corroborating the known pro-homeostatic role of the endocannabinoid system in other tissues [8]. UVB irradiation increases the levels of AEA and 2-AG in human keratinocytes [8]. Furthermore UVB increases TRPV1 expression in both keratinocytes and human skin *in vivo* [6] and increases COX-2 expression in cultured human keratinocytes [2] [14]. UVB radiation decreases keratinocyte viability, however the role of cannabinoid receptor ligands in UVB-induced keratinocyte cell death has not yet been studied. Therefore we evaluated in human cultured keratinocytes (HaCat), the effect of the most extensively studied endocannabinoid, AEA, and of synthetic cannabinoid receptor agonists and antagonists on cell survival in basal conditions and after acute UVB irradiation.

Materials and Methods

Materials

Dulbecco's minimal Eagle's medium (DMEM), antibiotic antimycotic solution, HEPES, anandamide (AEA), capsaicin (CAPS), capsazepine (CPZ) and indomethacin (IND) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Foetal bovine serum (FBS) and trypsin were purchased from Gibco (Paisley, UK). NaHCO₃ and EDTA were purchased from Merck (Germany). Arachidonoyl-2'-chloroethylamide (ACEA) was purchased from Biotrend Chemicals AG (Zurich, Switzerland) and AM251, AM630, JWH133 from Tocris Bioscience (Bristol, UK).

Cell line and culture conditions

Human HaCaT keratinocytes, provided by Prof. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany), were grown in DMEM supplemented with 10% FBS, 1% antibiotic antimycotic solution and 0.44 M NaHCO₃. Cells were maintained at <80% confluency, at 37°C in a 5% CO₂ (v/v), humidified atmosphere. HaCat cell cultures were incubated 2 h before the UVB irradiation with DMEM medium containing the different drugs studied and the medium was replaced every 24 h until the end of the experiment. Cannabinoid receptor agonists and antagonists were directly applied at a final ethanol concentration less than 0.3% (v/v). No significant influence of the vehicle was observed on the parameters determined.

UVB irradiation

HaCat cells in monoculture were washed with PBS and then exposed to acute UVB (312 nm) irradiation (70 mJ/cm²) emitted by a pre-heated, filtered, UV lamp (VL-6.M, Vilber Lourmat, France) at t = 0 min.

This was followed by a 48 h period of incubation after which the cells were harvested [10 min incubation with EDTA (0.05%, pH 7.4) and 5 min incubation with trypsin] for analysis.

The irradiation dose was defined according to UVB radiation cytotoxic effect. Non-irradiated controls were not exposed to UVB light, but subjected to all other manipulations. UV dosimetry was performed with a Waldmann 585.100 UV-meter (Herbert Waldmann GmbH & Co., Villingen-Schwenningen, Germany).

Cell viability determination

Harvested cells were centrifuged at 300 x g for 5 min at room temperature, washed twice with PBS and resuspended in 1 ml of PBS. From this

suspension, 180 µl were transferred into a black/clear bottom Microtest™ 96-well Assay Plate, Oplilux™ (BD Biosciences, Franklin Lakes, NJ, USA) for cell viability assay. Cell viability was measured using calcein-AM (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions. Each well containing 180 µl of cell suspension was loaded with 20 µl of calcein-AM (10 µM, in PBS solution). Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths using a SpectraMax, Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at 40 min incubation at room temperature.

Statistical analysis

Data are presented as means ± SEM. Student's t-test connected by the Newman Keuls method for multiple comparisons was used to analyze differences. Values of *P* < 0.05 are considered significant.

Results

Effect of anandamide on keratinocytes (HaCat) cell viability in basal conditions and after UVB irradiation

In basal conditions, we used AEA 3 and 30 µM and in the lowest concentration AEA (3 µM) slightly increased cellular viability while AEA 30 µM had no effect (Fig.1a). This effect of AEA (3 µM) was not reverted by CB₁ antagonist (data not shown).

Initially, we investigated the effect of different UVB irradiation doses (30-100 mJ/cm²) on keratinocytes cell viability (data not shown). We found that after high-dose (70 mJ/cm²) UVB irradiation a 60% reduction on cell viability was observed in control situation (Fig. 1a). AEA 30µM significantly increased (*P* < 0.05, n=12) UVB-induced cytotoxic effect (Fig. 1a).

To assess whether the metabolism of anandamide by COX-2 was necessary for endocannabinoid-induced cell death as previously proposed, cells were incubated with AEA and the COX-2 inhibitor, indomethacin. Indomethacin (1 µM) did not modify the AEA effect on cell viability in irradiated keratinocytes (fig.1b).

As anandamide can activate both cannabinoid receptors and TRPV1 we then investigated which "AEA-receptor" was mediating this effect of AEA. Cells were treated with AEA 30 µM and the CB₁ selective antagonist AM251 (1 µM) and the specific TRPV1 antagonist, capsazepine (5 µM). Figure 1b shows that CB₁ antagonist did not revert AEA effect. In contrast, capsazepine (5 µM), which alone did not modify cell viability, completely abolished

the increase in UVB-induced cell death by AEA (Fig.1b).

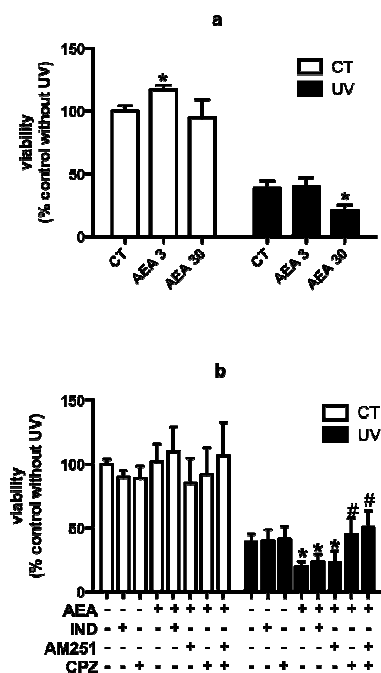


Figure 1. Effect of anandamide on keratinocytes (HaCat) cell viability in basal conditions and after UVB irradiation. Cell viability was evaluated before (CT) and after UVB (70 mJ/cm²) irradiation (UV) **a**) HaCat keratinocytes were treated for 48 h by vehicle (CT) and AEA (3-30 M) **b**) HaCat keratinocytes were incubated with AEA 30 M, COX-2 inhibitor, indomethacin (IND) 1μM, CB₁ selective antagonist, AM251 (1 M) and TRPV1 selective antagonist, capsazepine (CPZ) 5 M. Cell viability was expressed as the percentage of untreated controls, set to 100. Bars represent means ± SEM (*n* = 12) **P*<0.05 compared to respective control values. #*P*<0.05 compared to AEA 30 μM after UVB.

Effect of cannabinoid receptors and TRPV1 on UVB-induced keratinocytes cell death

In basal conditions neither the CB₁ selective agonist ACEA (1 μM) nor the CB₁ selective antagonist AM251 (1 μM) modified keratinocytes cell viability (Fig.2a). The CB₂ selective agonist JWH 133 (1-10μM) and the CB₂ selective antagonist AM 630 (1μM) had no effect (data not shown).

Neither the CB₁ selective agonist nor the CB₁ selective antagonist modified modified UVB-induced keratinocytes cell death (Fig. 2a).

As a positive control, we used the TRPV1 agonist capsaicin (10 μM), which had no effect in basal conditions but after UVB irradiation replicated the effect of AEA 30 μM increasing the UVB-induced

cytotoxic effect (Fig. 2b). This effect of capsaicin was also dependent on TRPV1 since it was completely reverted by capsazepine (Fig.2b).

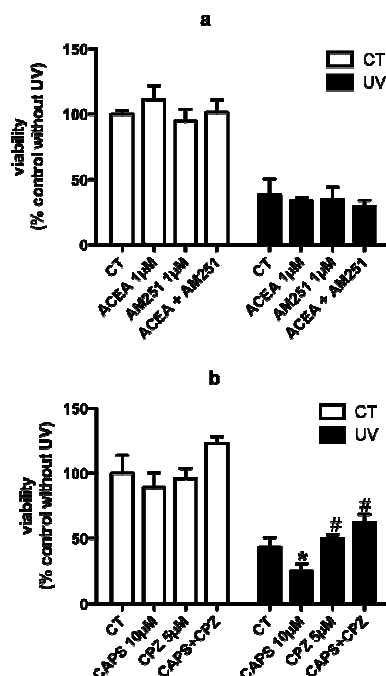


Figure 2. Effect of CB₁ receptor and TRPV1, in basal conditions and after UVB irradiation. Cell viability was evaluated before (CT) and after UVB (70 mJ/cm²) irradiation (UV). **a**) HaCat keratinocytes were incubated with CB₁ selective agonist, ACEA (1 M) and CB₁ selective antagonist AM251 (1 M) **b**) HaCat keratinocytes were treated for 48 h with TRPV1 agonist, capsaicin (CAPS) 10 M and TRPV1 selective antagonist, capsazepine (CPZ) 5 M. Cell viability results were expressed as the percentage of untreated controls set to 100. Bars represent means ± SEM (*n* = 12) **P*<0.05 compared to controls. #*P*<0.05 compared to CAPS 10 μM after UVB.

Discussion

In this study we provide evidence that the prototypic endocannabinoid AEA that is produced in keratinocytes after UVB irradiation [8] increases UVB-induced human keratinocytes cell death. We also demonstrated that this AEA effect is TRPV1 dependent. Anandamide produced by keratinocytes after UVB irradiation might act at TRPV1 in a paracrine or autocrine manner and negatively regulates their survival.

Acute UVB irradiation causes keratinocyte apoptosis and this programmed cell-death of UV-damaged skin cells is a definitive cancer-prevention pathway. Furthermore keratinocyte apoptosis after UVB irradiation is a key mechanism in psoriatic

plaques clearance after phototherapy [15]. Our data support the concept that the fine-tuned endogenous endocannabinoid tone of the skin could contribute to eliminate damaged keratinocytes after UVB irradiation.

In basal conditions, neither AEA nor the other CB₁ and CB₂ cannabinoid receptors agonists used suppressed keratinocyte cell viability. Indeed a slight increase of HaCat viability was observed with AEA at lower concentrations. This increase has already been described in a previous study [13]. By contrast, another research found that AEA, dose-dependently, inhibits proliferation and cell viability of human epidermal keratinocytes in culture [12]. However in that study a shorter incubation period with AEA was used and the effect of UVB radiation was not investigated. The authors proposed that AEA-induced keratinocyte death resulted from the sequential engagement of CB₁ receptor and TRPV1 with consequent calcium influx [12]. UVB increases TRPV1 expression in both keratinocytes and human skin in vivo [6] and this could explain that we only observed TRPV1-mediated AEA effect after UVB exposure. The need for higher concentrations of AEA to reduce cell viability after UVB radiation is consistent with the lower affinity of AEA for TRPV1 in comparison to CB₁, as clearly demonstrated in vitro through binding assays [10]. Furthermore the lack of effect of the CB₁ selective agonist ACEA also corroborates this TRPV1 dependent pathway. Previous studies have shown that metabolism of AEA by COX-2 was important for AEA-induced cell death in tumorigenic keratinocytes and that neither CB₁ nor CB₂ nor TRPV1 receptors were required for this response [13] [5]. Interestingly, in our model with non-tumorigenic HaCat cells, COX-2 inhibition by indomethacin did not modify the AEA effect in irradiated keratinocytes.

In conclusion, our results suggest that AEA through TRPV1 decreases cell viability in keratinocytes after ultraviolet exposure. Endocannabinoids participate in the adaptive skin response to UVB radiation and could be in the future useful in combination to phototherapy for the treatment of hyperproliferative dermatoses such as psoriasis.

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Effect of ultraviolet B radiation on endocannabinoid metabolizing enzymes

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Introduction Endocannabinoids are produced on demand and are rapidly cleared by a process of cellular uptake, followed by enzymatic metabolism. There is general consensus that the enzyme fatty acid amide hydrolase (FAAH) is the key-enzyme of the breakdown of anandamide, On the other hand, monoacylglycerol lipase (MAGL) is the major contribution for 2-AG metabolism.

Aim To evaluate the influence of ultraviolet B (UVB) radiation on endocannabinoid enzymes in the three models of skin cell cultures.

Methods Human SK-mel-1 melanoma cells (ATCC no. HTB-67), human HaCat keratinocytes, provided by Prof. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany), were used. SK-mel-1 and HaCat cells, ratio 1.8:2, were co-cultivated. Cells in co-culture were exposed to high (7 mJ/cm²) dose of UVB light wavelength (312 nm). SK-mel-1 cells in monoculture were exposure to UVB light under the same conditions as the cells in co-culture. HaCat cells in monoculture were exposed to high (60 mJ/cm²) dose UVB light. The irradiation doses were defined according to cell type based on the UVB radiation effect on cell viability. Non-irradiated controls were not exposed to UVB light, but subjected to all other manipulations. MAGL activity and FAAH activity were measured as previously described (De Petrocellis L *et al.*, 2011).

Results

Basal activity of both MAGL and FAAH was higher in HaCat cells than in SK-mel cells (V_{max} ; 84.13 and 16.65 versus 45.7 and 2.08 pmol/min/mg, respectively).

Table 1- Influence of UVB irradiation on the activity of endocannabinoid metabolizing enzymes. Results expressed in percentage of the respective control value.

		MAGL (% control)	FAAH (% control)
Sk-mel-1	CT	100	100
	High UVB dose	103.8	159.6
HaCat	CT	100	100
	High UVB dose	135.0	108.1
Co-cultures	CT	100	100
	High UVB dose	94.3	130.4

Conclusions The metabolizing enzymes in keratinocytes respond to UVB with an increase of MAGL activity whereas in melanoma cells and co-cultures only FAAH activity increases.

De Petrocellis L *et al.* Br J Pharmacol. 2011 Aug;163(7):1479-94

ORIGINAL ARTICLE

Ultraviolet B radiation differentially modifies catechol-*O*-methyltransferase activity in keratinocytes and melanoma cells

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Summary

Key words:

catechol-*O*-methyltransferase; cell death; keratinocytes; melanoma; tolcapone; ultraviolet radiation

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None declared.

Background: Catechol-*O*-methyltransferase (COMT) is a ubiquitous enzyme inactivating catecholic compounds. COMT is expressed also in human skin samples, and in melanoma cells it may be cytoprotective. A role of COMT in keratinocytes (HaCat) is unknown.

Objective: The objective of this study is to investigate whether ultraviolet-B (UVB) radiation modifies COMT activity in melanocytes and HaCat and whether COMT inhibition plays a role in UVB-induced cell death.

Methods: Human cell lines of melanotic melanoma (SK-mel-1) and HaCat were used. COMT activity was evaluated under basal conditions and after UVB irradiation (311 nm) at a low (8 mJ/cm²) and a high dose (60 mJ/cm²). Tolcapone 1 μM was used to inhibit COMT.

Results: Both SK-mel-1 and Ha-Cat cells express COMT activity. In SK-mel-1, COMT activity is reduced nearly 50% both 24 h and 48 h after a high dose UVB. In Ha-Cat cells, COMT activity increased 24 h after a high dose UVB but decreased at 48 h. Tolcapone increases significantly the cytotoxic effect of high dose UVB irradiation only in HaCat. High concentrations of tolcapone reduced melanin levels in melanoma cells parallel to reduced cell numbers.

Conclusions: Ultraviolet radiation differentially modifies COMT activity in melanoma cells and HaCat. Furthermore, tolcapone increased death of HaCat after irradiation but did not affect melanoma cells.

Keratinocytes (HaCat) and melanocytes are exposed to numerous environmental insults, including ultraviolet (UV) radiation, against which they form the body's first line of defense. UV radiation, particularly its ultraviolet-B (UVB) component, initiates complex molecular processes, resulting in tanning, inflammation, apoptosis, and skin cancer. In skin cells, UVB exposure produces reactive oxygen species (ROS) and quinones resulting in several types of DNA and lipid components damage (1). Skin is endowed with a variety of enzymes and oxidative stress-specific pathways, modified following UV exposure that can reduce cell damage (2). Catechol-*O*-methyltransferase (COMT) is a ubiquitous enzyme that catalyzes the transfer of a methyl group from *S*-adenosylmethionine (SAM) to endogenous and exogenous substrates containing a catechol moiety (3). Catecholic compounds may be oxidated to quinones and again reconverted to the original catechols. This redox cycling process produces potentially hazardous free radicals that are all substrates of COMT (3). It has been demonstrated by different authors that COMT is present in melanocytes and that, during melanin synthesis, COMT may

reduce the formation of reactive quinones and therefore being considered as one of the protective mechanisms of melanocytes (4–6). The association of COMTval158met polymorphism with low COMT activity with an increased risk of vitiligo in Chinese population underscores this protective role of COMT (7). More recently, a high mRNA expression level of COMT was found in a reconstructed epidermis model prepared from human HaCat, and a detoxifying effect was speculated, but the role of the enzyme in HaCat has not been studied yet (8). Although UV radiation is the major environmental stress to the skin, and COMT is suggested to play a cytoprotective role, the influence of UV radiation on COMT activity and the effect of COMT inhibition on UV-induced cell death are unknown.

In order to investigate the influence of UV radiation on COMT activity and the effect of COMT inhibition on UV-induced cell death, we chose two models of cell cultures, one with human melanotic melanoma cells and the other with human HaCat. COMT activity and cell viability in the absence and in the presence of a COMT inhibitor (tolcapone) were evaluated before and after two irradiation doses of UVB (cytotoxic and noncytotoxic).

Materials and methods

Materials

Minimum essential medium Eagle (MEM), antimycotic solution, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and synthetic melanin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Tolcapone was synthesized at laboratory of Chemistry, Department of Research and Development, BIAL (Porto, Portugal). Fetal bovine serum (FBS) and trypsin were purchased from Gibco (Paisley, UK). NaHCO₃ and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany).

Cell line and culture conditions

Human SK-mel-1 melanoma cells (ATCC n°. HTB-67) were grown routinely in MEM, supplemented with 10% FBS, 1% antimycotic solution, 25 mM HEPES, and 0.26 M NaHCO₃, at 37°C in a 5% CO₂ (v/v) humidified atmosphere. Human HaCat, provided by Prof. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany), were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 1% antimycotic solution, and 0.44 M NaHCO₃. Cells were maintained at < 80% confluency, at 37°C in a 5% CO₂ (v/v), humidified atmosphere.

For experiments without irradiation, cultures were incubated for 48, 72, and 96 h with MEM medium containing tolcapone (0.01–100 µM), and the medium was replaced every 24 h until the end of the experiment. COMT inhibitor was directly applied at a final dimethyl sulfoxide (DMSO) concentration less than 0.01% (v/v). No significant influence of the vehicle was observed on any of the parameters determined.

Melanin determination

The melanin content of cells was determined according to Rad et al. (9). Briefly, cells were centrifuged at 300×g for 5 min, resuspended in 500 µl of a NaOH (1 M, in 10% DMSO) solution and incubated at 80°C for 2 h. This was followed by centrifugation at 500×g for 10 min, and the resulting supernatant was used for measurement of melanin absorbance at 420 nm in a PowerWave™ HT microplate scanning spectrophotometer (BioTek Instruments, Inc., Winooski, Vermont, USA). Synthetic melanin was used to construct a standard curve for the range of 1–100 µg/ml.

Cell viability and protein content determination

Harvested cells were centrifuged at 300×g for 5 min at room temperature, washed twice with phosphate-buffered saline (PBS) and resuspended in 1 ml of PBS. From this suspension, 180 µl were transferred into a black/clear bottom Microtest™ 96-well Assay Plate, Oplilux™ (BD Biosciences, Franklin Lakes, NJ, USA) for cell viability assay. Cell viability was measured using calcein-AM (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions. Each well containing 180 µl

of cell suspension was loaded with 20 µl of calcein-AM (10 µM, in PBS solution). Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths using a SpectraMax, Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at 40 min incubation at room temperature.

The protein content in the samples was determined by the method of Bradford (10) with bovine serum albumin as standard.

UVB irradiation

Melanocytes in culture were washed with PBS and then resuspended in 1 ml of PBS and returned to the plate well for exposure to low (8 mJ/cm²) and high (60 mJ/cm²) doses of UVB light (312 nm), emitted by a preheated, filtered, UV lamp (VL-6.M, Vilber Lourmat, Marne-La-Vallée, France) at t = 0 min. This was followed by 24 or 48 h incubation period after which the cells were washed with PBS. HaCat cells in monoculture were washed with PBS and then exposed to low (8 mJ/cm²) and high (60 mJ/cm²) dose UVB light at t = 0 min.

The low and high irradiation doses were defined according to UVB radiation effect on cell viability. With low UVB doses, cell viability was unchanged; by contrast, the high doses produced a cytotoxic effect. Nonirradiated controls were not exposed to UVB light but subjected to all other manipulations. UV dosimetry was performed with a Waldmann 585.100 UV-meter (Herbert Waldmann GmbH & Co., Villingen-Schwenningen, Germany).

For experiments with UVB and COMT inhibition, cultures were incubated for 24 and 48 h with MEM medium containing tolcapone (1 µM), and the medium was replaced every 24 h until the end of the experiment. Tolcapone (1 µM) was present in incubation medium 1 h before irradiation.

COMT assay

COMT activity was evaluated by the ability to methylate adrenaline to metanephrine, as previously described (3). Aliquots of 100 µl of enzyme preparation were preincubated for 20 min with 80 µl of phosphate buffer (5 mM); thereafter, the reaction mixture was incubated for 5 min with increasing concentrations of adrenaline (0.1–30 µM) in the presence of a saturating concentration of the methyl donor (SAM, 100 µM), the incubation medium contained also pargyline (100 µM), MgCl₂ (100 µM) and EGTA (1 mM). The preincubation and incubation were carried out at 37°C, in conditions of light protection, with continuous shaking and without oxygenation. In experiments conducted with the aim of studying the effect of the COMT inhibitor, the reaction mixture was preincubated for 20 min with adrenaline 30 µM and increasing concentrations of tolcapone. K_M and V_{max} values for COMT activity were calculated from nonlinear regression analysis using the GraphPad Prism statistics software package (version 5.0; GraphPad Software, La Jolla, California, USA). For the calculation of the IC₅₀, the parameters of the equation for one site inhibition were fitted to the experimental data.

For irradiation experiments COMT activity was evaluated 24 and 48 h after UVB irradiation (low and high dose), and the medium was replaced every 24 h until the end of the experiment.

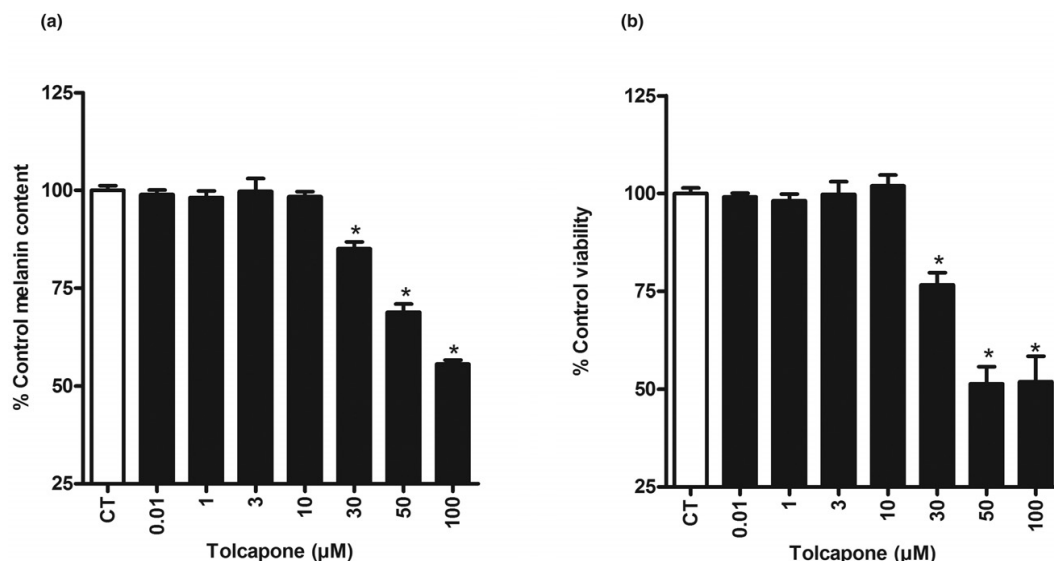


Fig. 1. (a) Cellular melanin content and (b) cell viability in SK-mel-1 cells after 96 h incubation with increasing concentrations of tolcapone (0.01–100 μM). Melanin and cell viability were determined in total cell extracts and expressed as the percentage of untreated controls. Bars represent means \pm SEM of eight experiments. * $P < 0.05$ compared with respective control values. SK-mel-1, melanocytes, SEM, standard error of the mean.

Na⁺, K⁺-ATPase assay

Na⁺-K⁺-ATPase activity in melanoma cells was used to exclude a nonspecific effect of UVB irradiation on cellular enzymatic activity. The activity was measured by the method of Quigley & Gotterer (11), with minor modifications. Briefly, melanocytes were permeabilized by rapid freezing, in liquid nitrogen, and thawing. The reaction mixture contained (in mM): buffer [imidazole 37.5, NaCl 75, KCl 5, sodium EDTA 1, MgCl₂ 5, NaN₃ 6, tris(hydroxymethyl)aminomethane(tris)hydrochloride 75] and 100 μl cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mM ATP. For determination of ouabain-insensitive adenosine 5'-triphosphate (ATPase), NaCl and KCl were omitted, and ouabain (1 mM; 100 μl) and vehicle (water; 100 μl) were added to the assay. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 50 μl of ice-cold trichloroacetic acid. Samples were centrifuged (1500×g), and liberated P_i in supernatant was measured by spectrophotometry at 740 nm. Na⁺, K⁺-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-insensitive ATPase.

Statistical analysis

Data are presented as means \pm standard error of the mean or 95% confidence intervals. Analysis of variance was used to analyze differences. Values of $P < 0.05$ are considered significant.

Results

Incubation of melanocytes (SK-mel-1) and HaCat homogenates in the presence of increasing concentrations of adrenaline resulted in a concentration-dependent formation of metanephrine. We showed that SK-mel-1 and HaCat cells express COMT activity [$V_{\max} = 8.91 \pm 0.29$ nmol/(mg protein)/h; $K_M = 0.78$ (0.52–1.03) μM] and [$V_{\max} = 4.43 \pm 0.22$ nmol/(mg protein)/h; $K_M = 4.65$ (3.14–6.17) μM], respectively. Tolcapone produced a concentration-dependent decrease in COMT activity with an IC₅₀ value of 4.3 (2.8–6.4) nM for SK-mel-1 and 6.3 (2.0–19.6) nM for HaCat cells.

In our culture model, time-course experiments (48, 72 and 96 h) showed that tolcapone (0.01–10 μM) had no influence on melanin content but 30–100 μM reduced it dose dependently (Fig. 1a). Although this effect was already observed after 72 h (data not shown), it was more evident after 96 h (Fig. 1a), and it was associated with a reduction in cell viability (Fig. 1b). Two well-known melanogenesis inhibitors, kojic acid (1 mg/ml) and hydroquinone (40 μg/ml), were used as positive controls and both had a significant cytotoxic effect similar to tolcapone 30 μM and 100 μM, respectively (data not shown).

COMT activity in HaCat increased 24 h after high dose UVB irradiation (60 mJ/cm²) but decreased 48 h after this irradiation (Fig. 2b). By contrast, in melanoma cells, COMT activity has already decreased 24 h after high dose irradiation (Fig. 2a) when no cytotoxic effect of UVB was present (Fig. 3a). Na⁺-K⁺-ATPase

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Fig. 2. COMT activity 24 h and 48 h after UVB irradiation with low (8 mJ/cm²) and high (60 mJ/cm²) doses in SK-mel-1 (a) and HaCat (b). The concentration of adrenaline used was 30 μ M. Bars represent means \pm SEM of eight experiments. $P < 0.05$ compared with respective control values. COMT, catechol-O-methyltransferase; CT, control; UVB, ultraviolet-B; SK-mel-1, melanocytes; HaCat, keratinocytes; SEM, standard error of the mean; UV, ultraviolet.

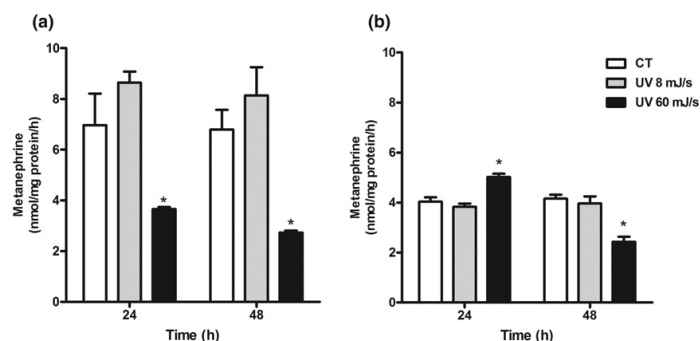
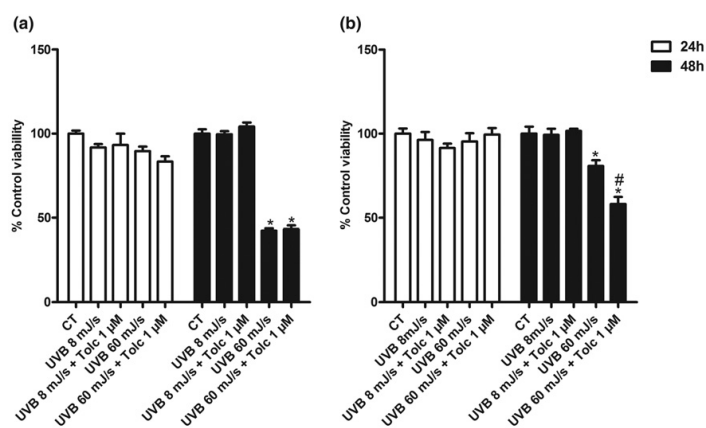


Fig. 3. Effect of COMT inhibition by tolcapone (1 μ M) in cell viability 24 h and 48 h after UVB irradiation with low (8 mJ/cm²) and high (60 mJ/cm²) doses in SK-mel-1 (a) and HaCat (b). Cell viability results are expressed as the percentage of untreated controls. Bars represent means \pm SEM of eight experiments. * $P < 0.05$ compared with respective control values, # $P < 0.05$ compared with respective irradiation dose without tolcapone. COMT, catechol-O-methyltransferase; CT, control; UVB, ultraviolet-B; SK-mel-1, melanocytes; HaCat, keratinocytes; SEM, standard error of the mean.



activity in melanoma cells excluded a nonspecific effect of UVB irradiation on cellular enzymatic activity since, after UVB irradiation in the same conditions, the activity of this enzyme did not change and the results [in nmol/(mg protein)/min] were 35.38 ± 1.18 , 29.67 ± 2.73 , and 36.13 ± 4.29 , in control situation, 24 h and 48 h after high dose UVB irradiation, respectively.

Tolcapone (1 μ M) did not change cell viability of melanoma cells after UVB irradiation (Fig. 3a), but it decreased significantly the viability of HaCat at 48 h after a high dose UVB irradiation (Fig. 3b).

Discussion

The results presented here show that 24 h after high dose UVB irradiation, COMT activity was significantly increased in HaCat, but it was decreased in melanoma cells. The effect in melanoma cells persisted 48 h after and was not a nonspecific effect on cellular enzymatic activity since the Na⁺-K⁺-ATPase activity was not modified. On the other hand, the effect in HaCat was inverted 48 h after irradiation, when a reduction in COMT activity was observed.

As expected, high dose UVB irradiation had a cytotoxic effect in both HaCat and melanoma cells, but tolcapone significantly

increased this UVB-cytotoxic effect only in HaCat. Although an effect of tolcapone itself cannot be excluded, this response may suggest a protective role of COMT in this skin type cell. UV radiation results in an increase generation of ROS that overwhelms the antioxidant defense mechanisms of the target system (1). COMT might be one of the several enzymes that modulate this oxidative stress in HaCat.

The single COMT gene codes for two separate enzymes are soluble (S-COMT) and membrane-bound (MB-COMT) forms. Both COMT isoforms have been reported to be widely distributed in the brain and peripheral tissues; however MB-COMT, is more prevalent in the brain tissues and the highest amount of S-COMT is located in the liver, duodenum, and kidneys (12). The two isoforms of COMT are proposed to have at least partially distinct roles; in according with these different tissue distributions, MB-COMT is believed to be mainly responsible for O-methylation of neurotransmitters; on the other hand, S-COMT is thought to have a main detoxification role (12). Despite the two COMT forms were not separated, we found much lower Km values for COMT activity in melanoma cells than in HaCat and we can speculate that MB-COMT is the predominant isoform in melanoma cells and that S-COMT is more important in HaCat. These data are in agreement with previous studies reporting that

a substantial part of COMT activity in melanocytes was represented by the membrane-bound enzyme (4). Since melanocytes are considered as 'neurons of the skin', this distribution is not surprising. On the other hand, as HaCat are the main barrier to the transmission of UVB to the underlying melanocytes, they have several mechanisms to protect themselves from the toxicity of UV radiation. The immediate increase in COMT activity that we observed may represent one of these mechanisms. Corroborating this hypothesis is the increase of UVB-cytotoxic effect when COMT activity was inhibited by tolcapone. Since this effect was not observed in melanoma cells, we could hypothesize that different COMT isoforms may have different responses to UVB irradiation and consequently different roles in UVB-cell protection but this needs further investigation.

Although it has been described in the past an induction of cytotoxicity in melanoma cells through inhibition of COMT (13) and it was speculated a potential role for COMT in melanogenesis (5), in our melanoma cell line we did not find these effects. Tolcapone in concentrations up to 10 μ M (IC_{50} value was 4.3 nM) did not modify either cell viability or melanin content, and the effect observed with higher concentrations can be explained by the drug toxicity (14). The rate-limiting step in melanogenesis is the oxidation of tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) by the enzyme tyrosinase, and since it has been shown that skin tyrosinase activity is significantly increased in hairless COMT-deficient male pups, an increase in melanin synthesis could be expected (15). This potential role of the O-methylation of L-DOPA by COMT in the regulation of melanogenesis was also suggested in the paper by Axelrod *et al.* (16) and substantiated by the finding that highly pigmented melanomas show less COMT activity than low pigmented melanomas (17). In our experimental conditions, melanin levels were not altered with COMT inhibition, although we used a melanoma cell line instead of a primary human melanocyte culture and that may constitute a limitation in extrapolating our results to normal skin physiology. On the other hand, it should be noted that melanoma cell lines have been extensively used to study melanogenesis and UV radiation effects (9, 18).

Conclusion

Tolcapone increased death of HaCat after irradiation but did not affect melanoma cells, and although this suggests a protective role for this enzyme in HaCat, a direct effect of tolcapone cannot be excluded. Furthermore UVB, radiation differentially modifies COMT activity in HaCat and melanoma cells.

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Chapter II

Ultraviolet B radiation on patients with psoriasis

*Catechol-O-methyltransferase activity is higher in psoriasis patients
and is down-regulated by narrowband ultraviolet B treatment*

Eur J Dermatol 2012 in press

Narrowband ultraviolet B treatment for psoriasis increases serum vitamin A levels

Br J Dermatol 2012; 16(4):958-960

*Effect of narrowband ultraviolet B treatment
on endocannabinoid plasma levels in psoriasis patients*

Submitted for publication 2012

Catechol-*O*-methyltransferase activity is higher in psoriasis patients and is down-regulated by narrowband ultraviolet B treatment

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Abstract

Background –Narrowband ultraviolet B (nbUVB) phototherapy is widely used in psoriasis treatment. UVB irradiation decreases catechol-*O*-methyltransferase (COMT) activity in human keratinocytes and melanoma cells. COMT activity is higher in psoriatic lesions than in normal skin but the effect of nbUVB on COMT activity in patients with psoriasis is unknown.

Objectives - Evaluate COMT activity in patients with psoriasis and to determine whether nbUVB modifies this activity.

Methods - An open observational study was conducted with 20 psoriasis patients and 15 healthy volunteers. Patients were treated with nbUVB thrice weekly during six weeks and evaluated at baseline, three and six weeks after phototherapy and four weeks after stopping. In each evaluation body mass index (BMI), Psoriasis Area and Severity Index (PASI) and Dermatology Life Quality Index (DLQI) were calculated and blood samples for erythrocytes soluble (S-) COMT activity assessment were taken.

Results – Before phototherapy (baseline), using a single concentration of substrate adreneline (1000 μ M), S-COMT activity levels (pmol/mg protein/h) were significantly higher in psoriasis patients (41.95 \pm 16.14) than in controls (30.38 \pm 14.89). After nbUVB treatment for psoriasis, S-COMT activity significantly decreased to 34.43 \pm 9.18. This decrease was positively correlated with baseline activity. Four weeks after stopping phototherapy, S-COMT activity returned to levels identical to ones before treatment (baseline levels). After phototherapy, PASI score improved significantly but no correlation to baseline S-COMT values or decrease in S-COMT activity was found.

Conclusions - This study shows that baseline S-COMT activity is higher in psoriasis patients than in controls and that this activity is significantly decreased by nbUVB treatment for psoriasis. This decrease is more evident in patients with higher baseline S-COMT activity. **Key words** nbUVB, psoriasis, COMT

Introduction

Psoriasis is a chronic inflammatory disease of the skin that has been linked with psychiatric and cardiovascular co-morbidities although the underlying mechanisms remain unclear [1] [2]. The catechol-*O*-methyltransferase (COMT) enzyme degrades dopamine, noradrenaline, adrenaline, and other catechol-containing compounds and is dynamically regulated in response to environmental stimuli. The enzyme has been involved in various neurobiological functions including mood, cognition, stress response, pain, immunological response, cardiovascular control and fat distribution [3] [4] [5]. COMT activity in humans can be reliably measured in blood samples since its activity in erythrocytes reflects central and peripheral activities [6] [7]. The gene encoding the COMT enzyme has functional polymorphisms contributing to the interindividual variability in the

enzymatic activity [3]. In Chinese population, no significant association was found between the low COMT activity, COMTval158met polymorphism, and the risk of psoriasis [8].

COMT is expressed at high levels in human skin and increased COMT activity in lesional psoriatic skin compared with nonlesional skin or with skin of normal individuals has been reported, suggesting a role for catecholamines on psoriasis lesion [9] [10]. However the impact of psoriasis treatment on COMT activity has never been studied. We have recently shown that ultraviolet B (UVB) irradiation decreases COMT activity in human keratinocytes and melanoma cells [11]. Since narrowband UVB phototherapy (nbUVB) is widely used in psoriasis treatment the aim of the present study was to evaluate COMT activity in psoriasis patients and examine whether this activity is modified by nbUVB treatment.

Table 1. Characteristics among the 20 patients before starting phototherapy (baseline), three (W3) and six (W6) weeks after nbUVB phototherapy thrice weekly and one month after the last nbUVB exposure (W10).

	Baseline	W3	W6	W10
Sex, women/ men (%)	9(42.1)/ 11(57.9)	-	-	-
Age (Years)	50.5±13.4 (21-69)	-	-	-
BMI (Kg/m ²)	26.42±4.08 (18.00-34.20)	26.49±3.99 (17.90-33.80)	26.55±4.02 (18.20-33.90)	26.43±3.85 (18.20-33.10)
UVB total dose (J/cm ²)	0	6.62±0.53 (6.3-8.1)	21.82 ± 3.97 (14.0-29.1)	-
PASI	9.74±4.70 (5-20)	4.95±3.55* (1-14)	2.16±2.54** [#] (0-7)	2.84±3.17 (0-12)
DLQI	9.95±6.52 (1-23)	5.32±5.15* (0-17)	3.74±4.3** [#] (0-12)	3.42±4.19 (0-12)
COMT K _M (μM)	212±51	216±51	218±43	212±47
V _{max} (pmol/mg prot/h)	51.05±3.22	43.39±2.69	41.39±2.14 [#]	49.59±2.85***

values are mean±SD (range). BMI, body mass index; PASI, Psoriasis Area and Severity Index, DLQI; Dermatology Life Quality Index. *P<0.05 (W3 vs. baseline), **P<0.05 (W6 vs. W3), # P<0.05 (W6 vs. baseline),*** P<0.05 (W10 vs. W6).

Patients and methods

The regional ethical committee approved this open observational study and all participants gave informed consent. The inclusion criteria were patients with chronic plaque psoriasis, indication for phototherapy and free of psoriasis medication or other systemic therapy, at least 12 weeks before and during the study. Patients presenting psoriatic arthritis or other inflammatory diseases were excluded. We enrolled 20 patients with Fitzpatrick skin types II-III and 15 matched controls with no evidence of psoriasis or other systemic inflammatory disease. Subjects were admitted only in winter and early spring. Psoriasis severity was measured by Psoriasis Area and Severity Index (PASI) and Dermatology Life Quality Index (DLQI). To diminish subjectivity, the same dermatologist evaluated PASI. All psoriasis patients were evaluated before phototherapy (baseline), after three (W3) and six (W6) weeks of treatment and four weeks after stopping (W10). In each evaluation body mass index (BMI), PASI and DLQI were calculated and blood samples for red blood cell soluble S-COMT assay were taken in the morning, in a fasting state.

The nbUVB irradiation (311±2 nm) was administered using a Waldmann 7001 cabin (UVB-TL01; Waldmann Medizintechnik, Villigen-Schwenningen, Germany); the initial dose was 0.3-0.5 J/cm², according to patient's phototype, increasing 0.1 J/cm² in every session until a maximum of 2.0 J/cm², totalizing eighteen nbUVB exposures (thrice weekly, six weeks). Only eyes and genitalia were shielded during the irradiation.

S-COMT activity was determined according to the method of Schultz et al with minor modifications [12]. In brief, prewashed erythrocyte samples were hemolyzed with four volumes of ice-cold water. After vortexing, the samples were left standing on ice for 10 minutes and then centrifuged for 20 min at 4°C at 20,000 g. The supernatant was used for the S-COMT assay, which was carried out immediately after sample preparation. The incubation mixture contained 300 μL enzyme preparation, 375 μL incubation medium, and 75 μL 10 mM (final concentration 1 mM) adrenaline as the enzyme substrate. The final 750 μL reaction volume contained 100 mM sodium phosphate buffer (pH 7.8), 2 mM MgCl₂, and 200 μM S-adenosyl-L-methionine. The samples were incubated in a water bath at 37°C for 60 min. The tubes were transferred to ice, and the reaction was

stopped by adding 75 μL of ice-cold 2 M perchloric acid. After 10 min, the samples were centrifuged for 10 min at 4°C at 5400 g, and 500 μL aliquots of the supernatant filtered on 0.22- μm pore size Spin-X filter tubes (Costar) were used for the assay of metanephrine by means of high performance liquid chromatography with electrochemical detection. S-COMT activity was expressed as the amount of metanephrine formed (in pmol) per milligram of protein in the sample, per hour (pmol/mg prot/h), by the action of COMT on a single concentration (1000 μM) or on increasing concentrations (5-2000 μM) of the substrate adrenaline to perform kinetic studies.

The kinetic parameters, V_{max} (maximum velocity) and K_M (Michaelis-Menten constant) values were calculated from non-linear regression analysis by using the Graphpad Prism Software package (version 5.0). Data are presented as mean \pm SD and repeated measures of ANOVA followed by student's paired t test was used to determine the statistical significance. Values of $P < 0.05$ are considered significant. The correlation analysis was performed by calculating Spearman coefficient correlation.

Results

Baseline data and the effect of nbUVB for the different parameters studied in psoriasis patients are summarized in Table 1. Phototherapy significantly reduced PASI and DLQI scores ($P < 0.0001$) but did not change BMI. Before phototherapy (baseline) there was some interindividual variability of S-COMT activity but, using a single concentration of substrate adrenaline (1000 μM), mean values were significantly higher in psoriasis patients (41.95 ± 16.14 pmol/mg prot/h, ranging from 19.20 to 70.61) compared with controls (30.38 ± 14.89 pmol/mg prot/h, ranging from 12.39 to 54.65) (Fig.1). In psoriasis patients, nbUVB phototherapy significantly decreased ($P < 0.05$) COMT activity, resulting in a consistent decrease, to levels identical to controls, at the end of treatment (W6) (Fig.2). Four weeks after stopping phototherapy (W10), S-COMT V_{max} values returned to baseline levels (Table 1). Change in the enzyme activity was due to a variation on V_{max} values with no change in K_M values (Table 1). The magnitude of decrease in S-COMT activity after phototherapy ($\Delta\text{S-COMT}$) was positively correlated with enzyme activity before phototherapy ($P < 0.0001$; $R^2 = 0.76$). No significant correlation was found between baseline S-COMT or $\Delta\text{S-COMT}$ and the following variables: age, baseline BMI, baseline PASI, ΔPASI , baseline

DLQI, ΔDLQI , and cumulative nbUVB dose.

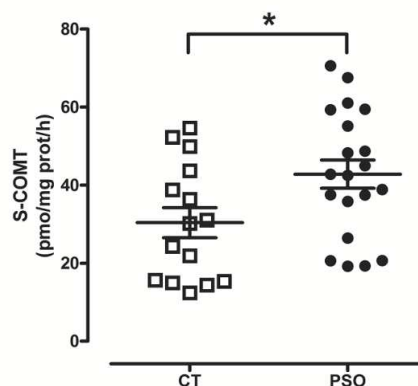


Figure 1. Red blood cell soluble (S-COMT) baseline activity (pmol/mg prot/h) in psoriatic patients ($n=20$) and in controls ($n=15$), using a single concentration of substrate adrenaline (1000 μM). Each circle represents a patient, * $P < 0.05$.

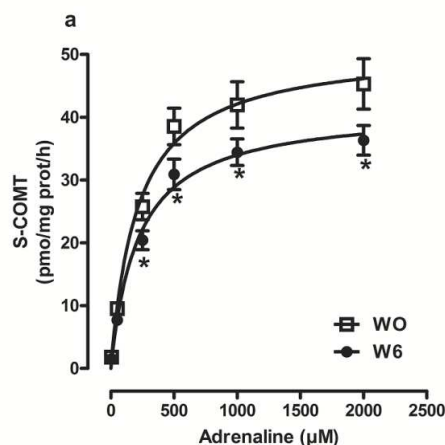


Figure 2. Red blood cell soluble (S-COMT) activity (pmol/mg prot/h) in psoriatic patients ($n=20$) before (baseline) and six (W6) weeks after nbUVB phototherapy thrice weekly, using increasing concentrations of the substrate adrenaline, * $P < 0.05$.

Discussion

The present study demonstrates that compared with controls, patients with psoriasis have significantly higher erythrocyte S-COMT activity. NbUVB phototherapy significantly decreased S-COMT activity, while clearing psoriasis. This UVB effect is in agreement with our data from human keratinocytes and melanoma cell lines where 48h after a high dose UVB (311nm) irradiation COMT

activity was significantly decreased in both cell types [11].

The previously reported lack of association between low activity COMTval158met polymorphism and psoriasis risk [8] is in agreement with the high erythrocyte S-COMT activity observed in our psoriasis patients. Furthermore, higher activity of COMT in lesional compared with nonlesional skin was observed in patients with psoriasis, suggesting a role for this enzyme in psoriasis [10]. It has also been reported that in several neurological cases treatment with levodopa, an important substrate of COMT, a significant improvement in accompanying psoriasis was observed and that in a study of nineteen cases of psoriasis treated with levodopa, most of them showed improvement [13] [14].

Phototherapy has been shown to induce apoptosis, inhibition of cell proliferation and immunosuppression but little is known about the in vivo effect of phototherapy at the enzymatic level. NbUVB reduces tumor necrosis factor α (TNF α) serum levels [15] and COMT expression is regulated by TNF α in a tissue-specific way [16] [17]. It can be speculated that the observed reduction in COMT activity might in part be related to the TNF α pathway.

COMT has attracted strong neuroscientific interest due to its central role in brain catecholamines signaling and has been linked to both depression and anxiety with contradictory results [4] [18] [3]. Patients with psoriasis are at increased risk for the development of depression, anxiety, and suicidality compared with the general population [1]. However the mechanisms by which psoriasis is associated with psychiatric outcomes are not completely understood and the role of COMT has never been studied. On the other hand, it is well known that phototherapy for psoriasis results in improved well-being and we hypothesize that phototherapy-induced decrease in COMT activity could be involved [19].

We found a positive correlation between baseline COMT activity and the magnitude of nbUVB-induced decrease in COMT activity. Despite not having found a correlation with clinical response the hypothesis that COMT activity could be related with therapeutic response to nbUVB treatment should not be excluded. Keratinocytes express beta 2 adrenergic receptors and produce adrenaline [20]. It has been proposed that the beta 2 adrenergic receptor activation plays a major role in calcium homeostasis, differentiation and proliferation in

human keratinocytes [20]. More recently reduction of beta 2 adrenergic response in keratinocytes has been implicated in the pathogenesis of psoriasis reviewed in [21]. Considering the nbUVB-induced decrease in S-COMT in psoriasis patients and our previous data of UVB-induced decrease in COMT activity in human keratinocyte cell lines [11] we may speculate that nbUVB in some way improve the disease by reducing adrenaline metabolism and consequently increasing beta2 adrenergic response in keratinocytes. Although erythrocyte S-COMT is considered a good indicator of COMT activity in other tissues, the effect of nbUVB on cutaneous COMT activity in psoriasis patients should be addressed. It was previously described [22] higher levels of cutaneous COMT activity in vitiligo patients than in healthy controls but such differences were not found in erythrocyte S-COMT activity. Taken together our results from psoriasis erythrocyte S-COMT activity and Bamshad et al [10] data from COMT activity in lesional psoriatic skin, we could suggest that contrarily to vitiligo, in psoriasis both erythrocytes and cutaneous COMT activities are higher than in controls.

This study has some limitations: it is an observational study and a direct causal relationship between COMT activity and psoriasis could not be established and healthy volunteers were not irradiated for obvious ethical reasons. The correlation between COMT activity, serum cytokine levels and the incidence of psoriasis comorbidities remains to be evaluated in future studies.

Our data indicate that COMT activity is higher in patients with psoriasis and that nbUVB decreases S-COMT activity, particularly in those patients with high baseline levels, while clearing psoriasis. COMT participates in an adaptive response to nbUVB exposure, the clinical and psychological impact of this response needs to be further investigated.

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opment of new blisters. Azathioprine 50 mg daily was started as adjuvant therapy to create a corticoid-sparing regimen, but was discontinued soon because of gastrointestinal side-effects. Two months later, the patient achieved complete remission.

We, for the first time, identify laminin-332 antibodies as the cause of orf-induced pemphigoid. The mechanism involved in the relation between orf and pemphigoid is still unclear. There might be a homology between laminin-332 and virus proteins, leading to a cross-reactivity also known as molecular mimicry. We performed a protein-protein blast of all protein sequences of the four orf strains whose complete genomes are deposited at NCBI GenBank (NZ2, OV-SA00, OV-18A2 and D1701) against all seven transcript variants of laminin-332. Unlike the situation described for the torque teno virus and the 180-kDa bullous pemphigoid antigen⁹ we found no overlap that was the size of an epitope (6–7 amino acids). The largest overlap was restricted to only four consecutive amino acids. If molecular mimicry of orf underlies pemphigoid here then it probably is a conformational epitope, which would also account for it binding to native skin substrate but not on immunoblot. On the other hand, it has been suggested that orf could alter basement membrane proteins, making them more antigenic.³ Using knockout skin for future cases could demonstrate if laminin-332 is the consistently targeted antigen in orf-induced pemphigoid.

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Narrowband ultraviolet B treatment for psoriasis increases serum vitamin A levels

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MADAM, Vitamin A (retinol) is obtained from diet, stored mainly in liver and adipose tissue and released 'on demand'. It is primarily transported bound to the adipokine, serum retinol-binding protein-4 (RBP4).^{1,2} The skin contains retinol-metabolizing enzymes and stores significant amounts of retinol as retinyl esters (RE).^{2,3} Retinol regulates several cutaneous responses but little is known about the mechanisms that mobilize its stores.^{2,3}

Photodegradation of cutaneous retinol and RE has been observed following exposure to ultraviolet (UV) radiation (UVR), either UVA or UVB.^{3–5} This photolability suggests that circulating retinol might be affected by phototherapy. No differences were observed in plasma vitamin A levels in volunteers exposed to UVR,⁶ but this issue should be analysed further. Our aim was to examine whether narrowband UVB (NB-UVB) phototherapy for psoriasis modifies vitamin A balance.

Patients with psoriasis, indication for phototherapy, and free of medication at least 12 weeks before and during the study were included. Psoriasis severity was measured by Psoriasis Area and Severity Index (PASI) and Dermatology Life Quality Index (DLQI). All patients were evaluated before phototherapy (baseline), after 3 (W3) and 6 (W6) weeks of treatment and 4 weeks after stopping (W10). In each evaluation body mass index (BMI), PASI and DLQI were calculated and blood samples for serum vitamin A, 25-hydroxyvitamin D (vitamin D) and folic acid measurements were taken in the morning, in a fasting state.

The NB-UVB irradiation (311 ± 2 nm) was administered using a Waldmann 7001 cabin (UVB-TL01; Waldmann Medizintechnik, Villingen-Schwenningen, Germany): initial dose $0.3\text{--}0.5$ J cm⁻², increasing by 0.1 J cm⁻² every session up to 2.0 J cm⁻², thrice weekly for 6 weeks.

Serum retinol was measured by high-performance liquid chromatography; serum vitamin D and serum folate were analysed by competitive radioimmunoassay. Data are presented as mean \pm SD, and repeated-measures ANOVA followed by Student's paired t-test were used to determine the statistical significance. $P < 0.05$ was considered significant. The correlation

Table 1 Characteristics of the 20 patients before starting phototherapy (baseline), at 3 (W3) and 6 (W6) weeks after narrowband ultraviolet B (NB-UVB) phototherapy thrice weekly and 1 month after the last NB-UVB exposure (W10)

	Baseline	W3	W6	W10
Sex, F/M, n (%)	8 (33)/12 (67)	—	—	—
Age (years), mean \pm SD (range)	51.2 \pm 13.4 (21–69)	—	—	—
BMI (kg m^{-2}), mean \pm SD (range)	26.3 \pm 3.9 (20.0–33.8)	26.2 \pm 3.8 (20.1–33.9)	26.3 \pm 3.8 (20.0–33.9)	26.3 \pm 3.9 (20.0–33.9)
NB-UVB total dose (J cm^{-2}), mean \pm SD (range)	0	6.5 \pm 0.5 (6.3–8.1)	21.9 \pm 6.1 (14.0–27.9)	—
PASI, mean \pm SD (range)	9.65 \pm 4.59 (5–20)	5.00 \pm 3.46* (1–14)	2.30 \pm 2.55** (0–7)	2.70 \pm 3.14 (0–12)
DLQI, mean \pm SD (range)	9.75 \pm 6.40 (1–23)	5.30 \pm 5.02* (0–17)	3.75 \pm 4.19** (0–12)	3.35 \pm 4.09 (0–12)
25-hydroxyvitamin D ($> 30 \text{ ng mL}^{-1}$) ^a , mean \pm SD (range)	18.44 \pm 12.56 (3–47)	24.35 \pm 13.00* (7–49)	34.05 \pm 12.24** (8–56)	34.61 \pm 12.50 (11–55)
Vitamin A ($> 1.05 \mu\text{mol L}^{-1}$) ^a , mean \pm SD (range)	1.62 \pm 0.34 (0.87–2.3)	1.76 \pm 0.46* (0.94–2.65)	1.86 \pm 0.44** (1.08–2.76)	1.97 \pm 0.53 (1.12–3.16)
Folate (2.2–17.5 ng mL^{-1}) ^a , mean \pm SD (range)	6.64 \pm 2.75 (3.2–14.6)	6.49 \pm 2.12 (3.5–10.8)	6.38 \pm 2.31 (3.7–11.4)	6.41 \pm 3.15 (2.4–15.5)

BMI, body mass index; PASI, Psoriasis Area and Severity Index, DLQI, Dermatology Life Quality Index. * $P < 0.05$ (W3 vs. baseline), ** $P < 0.05$ (W6 vs. W3). ^aReference intervals for biochemical parameters.

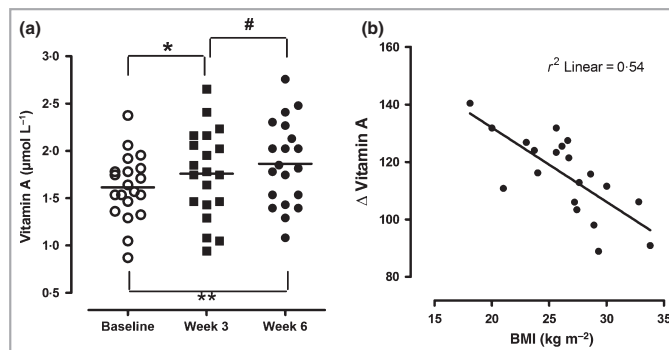
analysis was performed by calculating Spearman correlation coefficient.

Twenty patients completed the study. Baseline data and the effects of NB-UVB are given in Table 1. Phototherapy reduced PASI and DLQI scores, with 70% of the patients achieving PASI 75 (75% reduction in PASI compared with baseline) at W6 and a positive correlation between Δ PASI and Δ DLQI ($r = 0.25$; $P = 0.02$). Before treatment, 60% of the patients had insufficient serum vitamin D levels ($< 20 \text{ ng mL}^{-1}$), and these levels increased after phototherapy (Table 1). No correlation was found between this increase and the studied variables. At onset, all patients except one had normal serum retinol values ($> 1.05 \mu\text{mol L}^{-1}$), and these levels increased significantly after NB-UVB irradiation (Fig. 1a). The serum retinol remained ele-

vated at 1 month after the last treatment (W10) (Table 1). At baseline, vitamin A levels were not correlated with BMI but the percentage increase in vitamin A from baseline to W6 (Δ vitamin A) was negatively correlated with BMI ($P < 0.0001$; $r^2 = 0.54$) (Fig. 1b). No significant relationships were found between Δ vitamin A and the studied variables.

RE account for 85–90% of total epidermal vitamin A. Most are depleted by a single exposure to UVB,⁴ and are rapidly replaced by retinol uptake from dermal blood vessels.^{3–5} It was reported that the activity of the enzyme that esterifies retinol in epidermis increased to 167% at 2 days after UVB irradiation, suggesting a rapid influx of unesterified retinol.⁷ We showed that NB-UVB significantly and persistently increased serum retinol levels while clearing psoriasis. One explanation could be

Fig 1. (a) Serum vitamin A levels in patients with psoriasis ($n = 20$) before (baseline) and at 3 and 6 weeks after narrowband ultraviolet B phototherapy thrice weekly. Horizontal lines indicates means. ** $P = 0.0001$, * $P = 0.02$, # $P = 0.04$. (b) Correlation between body mass index (BMI) (kg m^{-2}) and the change in serum vitamin A levels following phototherapy. Each circle represents a patient and shows the BMI compared with the percentage of increase in serum vitamin A levels from baseline to week 6 (Δ vitamin A).



960 Correspondence

that phototherapy-induced cutaneous depletion of vitamin A increases its skin uptake from circulation and consequently increases mobilization of hepatic and fat stores. Despite this flux to the skin, mobilization of retinol stores may be disproportionate, contributing to the observed increase in serum levels. Only two studies have dealt with the relationship between UVR and serum retinol levels.^{6,7} One involving healthy subjects irradiated for 2 weeks with UVA or broadband UVB did not find variation, and the other with hairless mice showed a transient depletion of serum retinol a few days after a single broadband UVB irradiation.^{6,7} Our data indicate that in patients with psoriasis, vitamin A participates in an adaptive response to NB-UVB exposure; the physiological role of this response needs to be further investigated.

It is well documented that vitamin A and RBP4 are involved in lipid metabolism.¹ Subjects with nonalcoholic hepatic steatosis had lower retinol liver stores and an association between obesity and vitamin A deficiency was proposed.⁸ We found a negative correlation between BMI and Δ vitamin A and we can hypothesize that overweight patients have less adaptive vitamin A response.

In agreement with data from higher-latitude regions, we found a high prevalence of vitamin D insufficiency and a substantial benefit of NB-UVB.^{9,10}

The limitations of the present study include the absence of a control group, and cutaneous retinol and serum RBP4 levels should be addressed in future studies.

In addition to a significant increase of the vitamin D status, NB-UVB increases serum vitamin A levels while clearing psoriasis, the magnitude of this increase being determined by the patient's BMI.

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Effect of narrowband ultraviolet B treatment on endocannabinoid plasma levels in psoriasis patients

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ABSTRACT

Background- Psoriasis is associated with increased risk of obesity and metabolic syndrome. Although endocannabinoid system dysregulation was demonstrated in these comorbidities, no data exist on endocannabinoid plasma levels in psoriasis. We have recently shown that the cutaneous endocannabinoid system responds to ultraviolet B (UVB) irradiation, but the effect of narrowband UVB (nbUVB) treatment on plasma endocannabinoid levels is unknown.

Objectives- To evaluate endocannabinoid plasma levels in psoriasis patients. To determine whether exposure to nbUVB modifies these levels. To correlate plasma endocannabinoid levels with metabolic parameters and leptin levels.

Methods- An open observational study was conducted with 21 psoriasis patients and 15 matched controls. Plasma levels of anandamide, 2-arachidonoylglycerol (2-AG), oleylethanolamide (OEA) and palmitoylethanolamide (PEA) were recorded. Patients were evaluated at weeks 0, 3 and 6 of nbUVB phototherapy and 4 weeks after stopping the treatment. In each evaluation body mass index (BMI), Psoriasis Area and Severity Index (PASI) and Dermatology Life Quality Index (DLQI) were calculated and fasting levels of C-reactive protein (CRP), high sensitivity CRP (hsCRP), cholesterol, triglycerides, glucose and leptin were measured.

Results- Endocannabinoid plasma levels did not differ significantly between controls and psoriasis patients, in whom, before phototherapy, anandamide levels were positively correlated with hsCRP. The nbUVB treatment significantly decreased anandamide levels to an extent positively correlated with BMI. PASI score improved but no correlation with the decrease of anandamide levels was found.

Conclusions- The present study shows that nbUVB treatment significantly decreases anandamide plasma levels in patients with psoriasis in a manner that is stronger in patients with higher baseline BMI.

Introduction

The major endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG), are lipid mediators that are produced “on demand” from membrane phospholipid precursors in the central nervous system, peripheral organs, skin and adipose tissue ^{1,2}. Anandamide and 2-AG act through specific G-coupled cannabinoid receptors type 1 (CB₁) and type 2 (CB₂) ¹⁻². The biological activity of endocannabinoids is exerted in all organs and is not limited to the central nervous system as previously believed ^{3,4}. In the skin, a fully functional endocannabinoid system (ECS) has been identified ⁵. We have shown that this cutaneous ECS responds to ultraviolet B (UVB) irradiation ⁶. However the effect of narrowband UVB (nbUVB) phototherapy on endocannabinoid plasma levels in humans is still unknown. Several studies found a link between psoriasis, obesity, metabolic syndrome and serum levels

of certain adipokines ⁷⁻⁸. Obesity and metabolic syndrome are well known pathological conditions where endocannabinoid system dysregulation was demonstrated ^{9,2,10}. It has been suggested that ECS tone is overall increased in human obesity ¹¹. In obese patients, elevated endocannabinoid plasma levels were associated with coronary circulatory dysfunction ¹². The relationship between obesity and systemic inflammation has been described in psoriasis patients and considered detrimental ⁸. This interaction provides the inflammatory base of the ‘psoriatic march’ that describes the development of vascular complications in psoriasis ^{13,8}. Recent data support a link between the endocannabinoid system and obesity-associated inflammation ². Previous studies suggested that mediators released from the adipose tissue such as leptin and/or endocannabinoids are involved in the initiation and development of cardiovascular disease ¹². At present, the impact of endocannabinoids on

psoriasis is unknown. In this context, we aimed to evaluate endocannabinoid plasma levels in psoriasis patients, the influence of nbUVB phototherapy on these levels and their correlation with leptin levels and inflammatory and metabolic parameters. Since the two cannabinoid receptor-inactive and metabolically related anandamide congeners, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), were recently found to be dysregulated in obese rats¹⁴, and are known to activate receptors involved in inflammation, i.e. the nuclear peroxisome proliferator-activated receptor- α (PPAR- α) and transient receptor potential vanilloid type-1 (TRPV1) channels, we have also measured the plasma levels of these two lipid mediators.

Patients and methods

The regional ethical committee approved this open observational study and all participants gave informed consent. The inclusion criteria were patients with chronic plaque psoriasis, indication for phototherapy and free of psoriasis medication or other systemic therapy, at least 12 weeks before and during the study. Patients presenting diabetes, psoriatic arthritis or other inflammatory diseases were excluded. We enrolled 21 patients with Fitzpatrick skin types II-III patients and 15 age, sex and body mass index (BMI) matched controls. Subjects were admitted only in winter and early spring. They were advised to not make any nutritional or lifestyle changes that could interfere with the results during the treatment period. Psoriasis severity was measured by Psoriasis Area and Severity Index (PASI) and Dermatology Life Quality Index (DLQI). To diminish subjectivity, the same dermatologist evaluated PASI.

All psoriasis patients were evaluated before phototherapy (baseline), after three (W3) and six (W6) weeks of treatment and four weeks after stopping (W10). In each evaluation BMI, PASI and DLQI were calculated and fasting venous blood samples were taken in the morning, for anandamide, 2-AG, OEA and PEA measurement. The blood parameters shown in Table 2 were also assessed in the same conditions.

Plasma levels of anandamide, 2-AG, PEA and OEA were measured by isotope-dilution liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry as described⁹. Fasting plasma leptin was determined by ELISA kit (Millipore, Billerica, MA, USA), according to manufacturer instructions.

The nbUVB irradiation (311 ± 2 nm) was administered using a Waldmann 7001 cabin;

(UVB-TL01; Waldmann Medizintechnik, Villigen-Schwenningen, Germany); the initial dose, dependent on patient's phototype, was 0.3-0.5 J/cm², increasing 0.1 J/cm² in every session until a maximum dose of 2.0 J/cm², totalizing eighteen nbUVB exposures (thrice weekly, during six weeks). Only eyes and genital were shielded during the irradiation.

Data are presented as means \pm SD and ANOVA with repeated measures was used to determine the statistical significance. Values of $P < 0.05$ are considered significant. The correlation analysis was performed by calculating Spearman coefficient correlation.

Results

As shown in Table 1, patients and controls were not significantly different regarding age or BMI with a mean BMI > 25 Kg/m² (overweight) in both groups.

Endocannabinoid plasma levels did not differ significantly between controls and psoriasis patients (Table 1). Phototherapy significantly reduced PASI and DLQI scores ($P < 0.0001$) but did not change BMI (Table 1). We observed a significant positive correlation between the percentage of decrease in PASI (Δ PASI) and the percentage of decrease in DLQI (Δ DLQI) from baseline to week 6 ($R^2 = 0.25$; $P = 0.02$).

In psoriasis patients, before phototherapy, hsCPR levels, were positively correlated with BMI ($R^2 = 0.29$; $P = 0.02$) and LDL-cholesterol ($R^2 = 0.43$; $P = 0.004$) and negatively with HDL-cholesterol ($R^2 = 0.23$; $P = 0.04$). No correlation was observed between PASI score and hsCPR or CPR values.

As shown in Fig.1, anandamide plasma levels significantly decreased after completion of the nbUVB treatment ($P < 0.001$). We found that the percentage of decrease in anandamide from baseline to week 6 was positively correlated with BMI ($R^2 = 0.35$; $P = 0.006$) and with anandamide levels before phototherapy ($R^2 = 0.57$; $P = 0.0002$), but no correlation was found with PASI. No significant nbUVB-effect on the levels of the other endocannabinoid, 2-AG, or of OEA and PEA was found (Table 1).

Table 1. Characteristics and endocannabinoid levels of controls and psoriasis patients before phototherapy (Psoriasis baseline), three (W3) and six (W6) weeks after nbUVB phototherapy thrice weekly and one month after the last nbUVB exposure (W10).

	Controls	Psoriasis baseline	Psoriasis W3	Psoriasis W6	Psoriasis W10
Sex, women/men (%)	5 (33.3) /10(66.7)	8 (33.3) /13(66.7)	-	-	-
Age, Years	46.33±2.36	51.2±13.4	-	-	-
BMI (Kg/m ²)	25.6±4.2	26.3±3.9	26.2±3.8	26.3±3.8	26.3±3.9
UVB total dose (J/cm ²)	-	0	6.5±0.5	21.9±6.1	-
PASI	-	10.20±5.02	5.21±3.42*	2.42±2.57**	2.84±3.16##
DLQI	-	9.60±6.23	5.12±5.08*	3.84±4.29**	3.84±4.19##
AEA (pmol/ml)	2.55±0.35	3.19±0.49	2.56±0.28	1.90±0.12#	1.99±0.13##
2-AG (pmol/ml)	7.14±1.17	7.37±0.79	6.88±0.76	6.29±0.58	6.98±1.12
OEA (pmol/ml)	19.13±1.41	20.80±1.46	21.42±1.85	18.81±1.17	19.24±1.29
PEA (pmol/ml)	33.64±2.24	32.04±2.14	36.31±4.87	32.35±2.77	32.51±2.85

Values are means ± SD. BMI, body mass index; PASI, Psoriasis Area and Severity Index; DLQI, Dermatology Life Quality Index; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; OEA, *N*-oleylethanolamine; and PEA, *N*-palmitoylethanolamine *P<0.05 (W3 vs. baseline), **P<0.05 (W6 vs. W3), # P<0.05 (W6 vs. baseline), ## P<0.05 (W10 vs. baseline).

Table 2. Inflammatory parameters and lipid profile in psoriasis patients before starting phototherapy (Psoriasis baseline), three (W3) and six (W6) weeks after nbUVB phototherapy thrice weekly and one month after the last nbUVB exposure (W10).

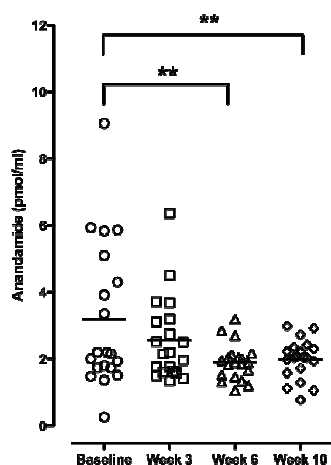
	Psoriasis baseline	Psoriasis W3	Psoriasis W6	Psoriasis W10
Total cholesterol (mg/dL)	212.70±40.66 (116-285)	211.10±40.35 (130-281)	212.40±37.71 (119-297)	206.90±43.08 (108-277)
LDL-cholesterol (mg/dL)	138.10±42.74 (77-214)	137.30±33.39 (88-202)	137.50±29.10 (89-214)	134.1±39.36 (73-201)
HDL-cholesterol (mg/dL)	52.89±10.10 (35-77)	52.13±11.24 (36-80)	51.71±10.12 (35-69)	54.00±11.25 (33-74)
Triglycerides (mg/dL)	136.30±74.85 (50-300)	137.70±69.30 (40-290)	137.10±63.50 (40-288)	134.00±59.98 (54-267)
Glucose (mg/dL)	87.74±19.10 (64-132)	88.76±21.88 (64-136)	85.27±23.31 (63-137)	90.67±19.65 (66-137)
Total Leptin (ng/mL)	10.46±7.19 (2.38-27.71)	11.24±6.27 (3.67-26.26)	10.19±5.78* (3.73-25.52)	12.13±7.41 (4.44-31.84)
Males Leptin (ng/mL)	10.19±7.02 (2.38-21.78)	10.96±5.47 (4.32-18.10)	9.53±4.72* (3.73-15.20)	11.60±5.95 (4.44-21.67)
Females Leptin (ng/mL)	11.37±8.05 (3.00-27.71)	11.40±7.81 (3.67-26.26)	11.01±7.36 (3.78-25.52)	12.72±9.17 (6.89-31.84)
CPR (<3.0 mg/L) ^a	2.53±1.74 (0.3-6.4)	2.37±1.77 (0.3-6.0)	2.82±1.91 (0.3-6.0)	2.68±2.00 (0.2-7.1)
hsCPR (<5.0 mg/L) ^a	2.24±1.61 (0.3-5.9)	3.55±4.41 (0.4-8.2)	2.96±2.83 (0.2-5.4)	2.79±2.74 (0.2-6.5)

Values are means ± SD (range). CRP, C-reactive protein; hsCRP, high sensitivity C-reactive protein. *P<0.05 (W6 vs. W3). ^a Reference intervals for biochemical parameters.

Table 3. Correlation of endocannabinoid plasma levels before phototherapy (anandamide, 2-AG, OEA and PEA) with clinical variables in patients with psoriasis

	AEA		2-AG		PEA		OEA	
variable	r	P-value	r	P-value	r	P-value	r	P-value
Age	0.20	0.39	-0.16	0.50	-0.04	0.85	0.16	0.51
PASI	0.005	0.98	0.30	0.20	-0.28	0.22	-0.18	0.45
DLQI	-0.31	0.18	-0.01	0.95	-0.44	0.05	-0.43	0.06
BMI	-0.36	0.11	0.23	0.32	-0.33	0.15	-0.37	0.09
Total cholesterol	-0.32	0.18	0.29	0.22	-0.45	0.05	-0.39	0.09
LDL-cholesterol	-0.35	0.14	0.12	0.63	-0.36	0.13	-0.29	0.24
HDL-cholesterol	-0.23	0.40	-0.08	0.74	-0.12	0.63	-0.09	0.73
Triglycerides	0.08	0.76	0.34	0.15	0.03	0.89	0.16	0.51
Glucose	0.29	0.25	0.005	0.99	-0.13	0.59	-0.19	0.43
Total Leptin	-0.02	0.94	0.56	0.02*	0.33	0.18	0.25	0.32
CPR	0.64	0.002*	-0.29	0.21	0.33	0.18	0.25	0.35
CPRhs	0.63	0.002*	-0.29	0.21	0.36	0.15	0.22	0.35

BMI, body mass index; PASI, Psoriasis Area and Severity Index; DLQI, Dermatology Life Quality Index; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; OEA, *N*-oleylethanolamine; and PEA, *N*-palmitoylethanolamine * $P < 0.05$

**Figure 1.** Plasma anandamide levels in psoriatic patients (n=21) before (baseline) and three and six weeks after nbUVB phototherapy thrice weekly. ** $P < 0.01$

Regarding to metabolic and inflammatory parameters, there was no significant effect of phototherapy (Table 2). Only a slight reduction of leptin levels was observed in males (Table 2).

A positive correlation was seen between baseline hsCPR and anandamide levels (Table 3). No correlation was found between baseline endocannabinoid, OEA and PEA levels and other clinical and metabolic parameters (Table 3).

Discussion

The present study provides evidence that nbUVB phototherapy in psoriasis patients decreases plasma levels of anandamide without changing 2-AG, OEA or PEA levels.

We observed that around 65% of the studied patients were overweight with body mass index (BMI) ≥ 25 and < 30 kg/m². Interestingly, the decrease in anandamide plasma levels was positively correlated with BMI at baseline. It was previously reported that increased anandamide plasma levels are present in women with binge eating disorder¹⁵ or post-menopausal obesity¹⁶. Furthermore, elevated circulating anandamide levels are associated with coronary circulatory dysfunction in obese individuals and proposed as a novel endogenous cardiovascular

risk factor¹². The observed UVB-induced anandamide decrease might, therefore, suggest a beneficial effect of phototherapy in psoriasis patients at cardiovascular risk. This hypothesis, which, however, needs further investigation, is supported by the observation that baseline anandamide levels were positively correlated with hsCRP, a biomarker for both increased cardiovascular events and inflammation. In another study¹², only anandamide plasma levels were correlated with CRP and an endocannabinoid-mediated association between metabolic and inflammatory cardiovascular disease mechanisms was proposed.

The link between psoriasis and increased cardiovascular risk appears to be dependent of disease severity¹⁷. A major limitation of our study was that patient profiles corresponded to those of candidates for phototherapy and, therefore, subjects with very severe cutaneous disease are not represented in the study population. This might explain why anandamide plasma levels were not significantly different between psoriasis patients and controls, also because the two populations did not differ for their BMI and were overweight ($\geq 25 \text{ kg/m}^2$)¹¹
16.

Despite the fact that the clinical response to UVB was good, no correlation was observed between endocannabinoid levels and disease activity. Furthermore, despite the recognized role of endocannabinoids in the metabolic syndrome, no correlation was observed between endocannabinoid levels and leptin, LDL cholesterol, HDL cholesterol, triglycerides or glucose fasting levels in our populations. Indeed, some of these correlations have been found so far only for obese patients (BMI ≥ 30) and/or only for 2-AG^{9,16}. Unlike 2-AG, anandamide, OEA and PEA are all *N*-acylethanolamines and share similar metabolic pathways¹⁸. Therefore, it was somewhat surprising not to find for OEA and PEA similar changes or correlations as with anandamide, especially considering the fact that for both compounds anti-inflammatory actions mediated by non-cannabinoid receptors have been reported¹⁹. This finding might suggest that the observed changes specifically reflect, or impact on, the activity of the endocannabinoid system, thus supporting their possible relevance to psoriasis- and cardiovascular-related inflammation¹⁸. However, the mechanism by which UVB might specifically alter anandamide remains to be explored.

It will be important to evaluate in the future the influence of other psoriasis treatments on endocannabinoid plasma levels in order to clarify whether the UVB-induced decrease of

anandamide plasma levels is a radiation effect *per se* or is related with disease remission.

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Discussion and Conclusions

Discussion

In **chapter I**, we report that keratinocytes and melanoma cells express CB₁ and CB₂ receptors, produce physiologically relevant concentrations of the two major endocannabinoids (2-AG and anandamide) and of the related *N*-acylethanolamine (PEA), and have the main cannabinoid metabolizing enzymes FAAH and MAGL. These findings show that these cutaneous cells are endowed with all the components of an endogenous cannabinoid system. Levels of endocannabinoids are much higher in melanoma cells than in keratinocytes, but FAAH activity and particularly MAGL activity are higher in keratinocytes. This much higher activity of MAGL in keratinocytes may explain why the basal levels of 2-AG are lower in co-cultures than in melanoma cells alone.

We have also demonstrated that UVB irradiation increases the levels of anandamide, 2-AG and PEA in keratinocytes and significantly increases the ratio 2-AG/anandamide in co-cultures whereas in melanoma cells endocannabinoid levels remain unaltered after UVB. These findings suggest that endocannabinoids are also pro-homeostatic in the skin, because UV irradiation is the major environmental insult to the skin and elicits an increase in the levels of endocannabinoids.

Since UVB increases Ca²⁺ influx in keratinocyte (HaCat) via TRPV1 activation (Lee *et al.* 2009; Masaki *et al.* 2009) and endocannabinoids are produced “on demand” from membrane phospholipids in response to intracellular Ca²⁺ (Alger and Kim 2011; Cluny *et al.* 2012), we can hypothesize that the observed increase in endocannabinoid levels is dependent on UVB-induced Ca²⁺-influx. Moreover, in human skin and keratinocytes, UVR activates phospholipases A₂, C and D and diacylglycerol kinase, thereby releasing arachidonic acid and DAG (De Leo *et al.* 1984; Punnonen and Yuspa 1992; Carsberg *et al.* 1995) which are the main precursors for endocannabinoid synthesis. The UVB-induced release of DAG, which is the 2-AG precursor, may explain why 2-AG increases more than anandamide in keratinocytes irradiated with

UVB. The increase in the ratio 2-AG/anandamide that we observed in co-cultures after exposure to UVB can also be explained by the UVB-induced release of DAG. Our data agree with the findings that mouse epidermal JB6P+ cells respond to stress factors such as UVB irradiation or serum deprivation with the production of increased levels of *N*-acylethanolamines and their precursors, *N*-acylethanolamine phospholipids (Berdyshev *et al.* 2000).

An alternative explanation for the increase in endocannabinoid levels after UVB irradiation would be a decrease in metabolizing pathways. However we observed that UVB even caused an increase of MAGL and FAAH activities. These results suggest that the UVB increased levels of endocannabinoids are mostly due to an increased synthesis.

Total endocannabinoids (except PEA) are less in co-culture than in melanoma cells alone under basal conditions, and even less under UVB irradiation. This phenomenon may be due to the high levels of endocannabinoid-degrading enzymes in keratinocytes and their up-regulation by UVB.

On the other hand UVB increases the mRNA expression of CB₁ receptors in melanoma cells but not in keratinocytes. Taken together, our data support the concept that endocannabinoids may participate in the important cross talk between keratinocytes and melanocytes in the epidermal unit after exposure to UVR. These results are in agreement with the classic idea that keratinocytes respond to UVR producing several paracrine factors that will act on melanocytes helping in their protection.

Endocannabinoids (Karsak *et al.* 2007) and PEA (Petrosino *et al.* 2009) attenuate the cutaneous allergic response whereas the antagonism of CB₁ cannabinoid receptors in human HaCat keratinocytes increases the expression of pro-allergic chemokines (Leonti *et al.* 2010). Acute UVB irradiation is known to cause immunosuppression. Since we found, in **chapter I**, that UVB irradiation is accompanied by up-regulation of both endocannabinoids and PEA levels, we hypothesize that the cutaneous

endocannabinoid system could play a role in UVB-induced immunosuppression and might contribute to the well-known therapeutic effect of UVB radiation.

Acute UVB exposure also causes inflammation and there is growing evidence suggesting that 2-AG modulates the inflammatory response by acting on the cannabinoid CB₂ receptor (Oka *et al.* 2006). Interestingly, we found a significant increase in 2-AG after UVB irradiation, suggesting a role for endocannabinoids, namely 2-AG, in UVB-induced inflammation. In agreement with this hypothesis, Zheng *et al.* (Zheng *et al.* 2008) suggested that CB receptors and the related signaling pathways might be involved in the promotion of *in vivo* UVB-induced skin inflammation. In CB₁ and CB₂ double gene-deficient mice, the authors observed a marked decrease in UVB-induced inflammation and an attenuation of UVB-induced activation of MAPK and NF- κ B. (Zheng *et al.* 2008). On the other hand, PEA with its anti-inflammatory effect (Petrosino *et al.* 2010) might represent an endogenous protective agent against UVB-induced inflammation.

The first defense against UVR is the cutaneous melanin produced in melanocytes. However, as reported in **chapter I** melanoma cells alone did not show any increase in melanin content after UVB irradiation. In contrast, co-cultures exhibited a marked melanogenic response to UVB irradiation (\approx 40% melanin over the controls). It is known that UVB irradiation of keratinocytes triggers the release of cytokines, growth factors, and other mediators such as POMC-derived peptides that regulate melanin synthesis in a paracrine way (Duval *et al.* 2001; Park *et al.* 2009) and this may explain the differences that we observed between mono- and co-cultures in their response to UVB.

In **chapter I**, we showed that the CB₁ selective agonist arachidonoyl-2-chloroethylamide (ACEA), had an inhibitory effect on basal melanogenesis in co-cultures. Furthermore, in the same model, ACEA abolished the increase in melanin content caused by UVB irradiation. The CB₁ selective antagonist (AM-251), that alone did not modify UVB-induced melanogenesis, reversed this inhibitory effect of ACEA.

The inhibitory effect of CB₁ receptor activation on melanogenesis was observed in co-cultures but not in monocultures, which again, underlines the important symbiotic relationship between melanocytes and keratinocytes. Since both cells express CB₁ receptors, it can be speculated that cannabinoids acting in keratinocytes inhibit the release of keratinocyte-derived paracrine melanogenic mediators. Alternatively, it is possible that CB₁ agonists directly act on melanoma cells to inhibit melanogenesis only when the conditions to produce melanin are optimized by the presence of keratinocytes. Our data suggest that human melanoma cells are both sources and, mainly, targets of endocannabinoids, which act as negative regulators of melanin synthesis after UVB radiation. The endocannabinoids produced by keratinocytes after UVB radiation might act at over-expressed melanocyte CB₁ receptors in a paracrine way, to counteract the excessive melanin production. From these results, we suggest that keratinocytes play a major role in cannabinoid-mediated inhibition of basal and, particularly, UVB-induced pigmentation. This could suggest a new endocannabinoid-mediated mechanism tightly regulating UVB-induced melanogenesis.

UVB irradiation evokes a signaling response through several pathways and is known to induce keratinocytes apoptosis as a mechanism to prevent malign transformation. The DNA damage-dependent pathway has been extensively investigated in recent years, but the underlying biochemical and enzymatic mechanisms are not completely understood. In **chapter I** we report that anandamide increases UVB-induced cell death in human keratinocytes through TRPV1 channel. Locally synthesized anandamide by keratinocytes, after UVB irradiation, might act at TRPV1 receptors in a paracrine or autocrine manner and reduces their survival. In basal conditions anandamide and the other cannabinoid agonists did not reduce keratinocyte cell viability. Recently anandamide has been found to inhibit proliferation and cell viability of human epidermal keratinocytes in culture by a sequential engagement of CB₁ receptor and TRPV1 channel (Toth *et al.* 2011). However in that study a shorter incubation period with anandamide was used and the effect of UVB

radiation was not investigated. Moreover UVB increases TRPV1 expression in both keratinocytes and human skin *in vivo* (Lee *et al.* 2009), which could explain that we observed TRPV1-mediated anandamide effect only after UVB exposure.

In addition, it was previously observed that metabolism of anandamide by COX-2 is important for anandamide-induced cell death in tumorigenic keratinocytes (Van Dross 2009; Kuc *et al.* 2012). Interestingly, in our model with non-tumorigenic HaCat cells, COX-2 inhibition by indomethacin did not modify the anandamide effect in irradiated keratinocytes.

Using the same experimental model, we decided to investigate other potential protective mechanisms against UVB irradiation. Despite the involvement of COMT in melanogenesis has been claimed in the past (Axelrod and Lerner 1963; Shibata *et al.* 1993), the role of this enzyme in keratinocytes and in UVB adaptive response had never been studied. In **chapter I**, we demonstrated that similarly to melanocytes, keratinocytes also have COMT activity and that acute UVB irradiation differentially modifies this activity in both cell types. Twenty-four hours after UVB irradiation, we observed a decrease in COMT activity in melanocytes while in keratinocytes there was an increase, but 48 h after UVB, the COMT activity decreases in both cell types. This COMT inhibition by UVB was not a nonspecific effect on cellular enzymatic activity since the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity was not modified. Furthermore the COMT inhibitor tolcapone increased the UVB-induced cell death only in keratinocytes. This response suggests that COMT has a protective effect against UVB-induced cell death in keratinocytes. Although, one cannot exclude that tolcapone has direct effects independent from COMT inhibition. Inhibition of COMT has been described to induce cytotoxicity in melanoma cells (Smit and Pavel 1995). It has also been proposed that COMT influences melanogenesis (Smit *et al.* 1994). In our melanoma cell line, COMT inhibition had no effect on cell viability nor on melanogenesis.

Afterwards we decided to study the effects of UVB radiation *in vivo* (**chapter II**). As previously mentioned, psoriasis is a common inflammatory skin disease

characterized by hyperproliferation of epidermal keratinocytes and it the prototypic disease showing a favourable response to nbUVB phototherapy. In addition to immunosuppression, keratinocyte apoptosis also occurs as a result of nbUVB (Aufiero *et al.* 2006; Weatherhead *et al.* 2011) and contributes to the beneficial effect of phototherapy in psoriasis. **In chapter I**, we report the effect of UVB irradiation in COMT activity *in vitro*, thus we decided to study COMT activity in patients with psoriasis treated with nbUVB (**chapter II**). For the *in vitro* studies the same wavelength (311nm) as in psoriasis phototherapy was used.

In agreement with our data from human keratinocyte and melanoma cell lines (**chapter I**) we demonstrated, in **chapter II**, that nbUVB phototherapy for psoriasis significantly decreases erythrocytes soluble COMT (S-COMT) activity.

UVB irradiation, of the skin is known to activate the NF-kB pathway (Chang *et al.* 2011) which regulates the COMT gene (Tchivileva *et al.* 2009). Since it was demonstrated that NF-kB transcription factor inhibits COMT expression in central nervous system (Tchivileva *et al.* 2009), we might speculate that this pathway could also be involved in UVB-induced COMT inhibition. Another explanation could be that COMT expression regulated by TNF α (Tchivileva *et al.* 2009; Tunbridge 2010) decreases because nbUVB reduces TNF α serum levels (Coimbra *et al.* 2010).

It was previously observed that the activity of COMT in patients with psoriasis is higher in lesional than in nonlesional skin and it was proposed that COMT might have a role in psoriasis (Bamshad *et al.* 1970; Barbeau and Giroux 1972). Interestingly, in **chapter II**, we found that patients with psoriasis have significantly higher S-COMT activity than controls. COMT has attracted strong neuroscientific interest due to its central role in brain catecholamines signaling and has been linked to both depression and anxiety with contradictory results (Aberg *et al.* 2011; Nyman *et al.* 2011; Witte and Floel 2011). Patients with psoriasis are at increased risk of depression, anxiety, and suicide compared with the general population (Kurd *et al.* 2010). The role of COMT in

psoriasis lesions and the potential relation between psoriasis, depression and COMT activity should be further explored.

In addition to psychiatric comorbidities (Kurd *et al.* 2010), it is well documented that psoriasis is associated with obesity and metabolic syndrome (McDonald 1989; Wakkee *et al.* 2007; Yiu *et al.* 2011). Co-occurrence of obesity and psoriasis could lead to interactions between the two diseases since adipokine disturbances are present in both (Coimbra *et al.* 2010; Gerdes *et al.* 2011).

We observed that around 70% of the studied psoriasis patients were overweight (body mass index (BMI) ≥ 25 kg/m²). Similarly to endocannabinoids, it has been recognized that retinol (vitamin A) and retinol binding protein 4 (RBP4), a recently described adipokine, are involved in lipid metabolism (Berry and Noy 2012). Recently it was observed that the CB₁ receptor gene transcription is up-regulated by retinol-derivate retinoic acid via retinoic acid receptor γ (RAR γ) (Svensson *et al.* 2006; Mukhopadhyay *et al.* 2010).

Retinol regulates several cutaneous responses and retinyl esters (RE) account for 85% to 90% of total epidermal vitamin A (Sorg *et al.* 2002). Cutaneous retinol and RE are depleted by a single exposure to UVB and rapidly replaced by retinol uptake from dermal blood vessels (Tran *et al.* 2001; Sorg *et al.* 2002; Fu *et al.* 2007). In **chapter II**, in order to clarify whether circulating retinol might also be affected by phototherapy we measured retinol in psoriasis patients treated with nbUVB. We demonstrated that nbUVB significantly and persistently increased serum retinol levels in psoriasis patients. One explanation could be that phototherapy-induced vitamin A cutaneous depletion increases skin uptake of retinol from circulation and consequently increases mobilization of hepatic and fat stores. It was reported that the activity of the enzyme that esterifies retinol in epidermis increased to 167% two days after UVB irradiation, which may cause a rapid influx of unesterified retinol (Torma *et al.* 1988). Despite its increased uptake by the skin, the mobilization of retinol stores may be disproportionate contributing to the observed increase in serum levels. We found a negative correlation

between BMI and this retinol increase and we hypothesize that overweight patients have less adaptive vitamin A response after nbUVB phototherapy.

As expected, we also identified a substantial benefit of nbUVB on vitamin D status but no correlation with the clinical response was found.

It is increasingly being recognized that in addition to obesity, psoriasis is associated with an increased cardiovascular risk (Wakkee *et al.* 2007; Yiu *et al.* 2011). Although a relationship between systemic inflammation of psoriasis and premature atherosclerosis was proposed (Yiu *et al.* 2011) the mechanisms remain poorly understood. The effect of psoriasis treatments on the development of cardiovascular and metabolic comorbidities has been a recent focus of awareness but the matter is far from clear. However the endocannabinoid system dysregulation has been implicated in the development of obesity and several cardiometabolic risk factors (Montecucco and Di Marzo 2012) and recent data support a link between the endocannabinoid system and obesity-associated inflammation (Cluny *et al.* 2012) no data exist on endocannabinoids plasma levels in psoriasis. In **chapter I**, we showed that UVB radiation modifies cutaneous endocannabinoid levels *in vitro* thus in **chapter II**, we studied, in psoriasis patients, the influence of nbUVB phototherapy in endocannabinoid (anandamide and 2-AG) plasma levels and its correlation with inflammatory mediators, metabolic parameters and leptin levels. Furthermore, since two cannabinoid receptor-inactive and metabolically related anandamide congeners, oleylethanolamine (OEA) and PEA are emerging as potent endogenous ligands of peroxisome proliferator-activated receptor alpha (PPAR α), a well established target for dyslipidemia, we have also measured the plasma levels of these two mediators. Phototherapy with nbUVB significantly decreases the plasma levels of the endocannabinoid anandamide while the levels of the other endocannabinoids remained constant. This decrease in anandamide plasma level was positively correlated with BMI. Increased anandamide plasma levels were previously reported to be associated with coronary circulatory dysfunction in obese individuals (Quercioli *et al.* 2011). The

observed UVB-induced anandamide decrease might suggest a beneficial effect of phototherapy on psoriasis patients cardiovascular risk, but this needs further investigation. Interestingly in psoriasis patients anandamide levels were positively correlated with high sensitivity C-reactive protein levels (hsCRP), a biomarker for increased cardiovascular events and inflammation.

Despite a good clinical response of psoriasis patients to UVB, no correlation was observed between endocannabinoid levels and disease activity. Although the recognized role of endocannabinoids on metabolic syndrome, no correlation was observed between endocannabinoid levels and leptin, LDL cholesterol, HDL cholesterol, triglycerides or glucose fasting levels.

Further studies are required to understand the cellular and tissue origin of plasma endocannabinoids in order to interpret the meaning of these UVB-induced changes. It would be important to evaluate in the future the influence of other psoriasis treatments, on endocannabinoid plasma levels in order to clarify whether the UVB- induced decrease of anandamide plasma levels is a radiation effect *per se* or is related with the disease remission itself.

Conclusions

1. Human keratinocytes and melanocytes cells have a fully functional endocannabinoid system. Cutaneous endocannabinoids participate in the well-known cross talk between keratinocytes and melanocytes.
2. UVB radiation up-regulates the cutaneous endocannabinoid system and activates a paracrine CB₁-mediated signaling that decreases melanin synthesis.
3. The major endocannabinoid anandamide increases UVB-induced cell death of human keratinocytes through TRPV1 receptors. Anandamide synthesized by keratinocytes after UVB irradiation acts at TRPV1 receptors in an autocrine manner and reduces their survival.
4. In our experimental conditions, melanin levels were not altered with COMT inhibition, contrary to the previously suggested role of *O*-methylation of L-DOPA by COMT in the regulation of melanogenesis.
5. UVB radiation decreases COMT activity in keratinocytes and melanoma cells. COMT inhibition by tolcapone increased UVB-induced cell death only in keratinocytes. Although this suggests a protective role for this enzyme in keratinocytes a direct effect of tolcapone cannot be excluded.
6. Patients with psoriasis have significantly higher S-COMT activity than controls. UVB significantly decreased this activity in patients as well as in human keratinocyte and melanoma cell lines. Further investigation is needed to explain the lack of correlation between COMT activity reduction and clinical response.
7. In addition to a significant increase of the vitamin D status, UVB radiation increases serum retinol levels in psoriasis patients. The degree of this UVB-induced increase in retinol levels is determined by the patients BMI.

8. Basal anandamide plasma levels, in psoriasis patients, were positively correlated with hsCPR levels. After UVB treatment a significantly decrease in the plasma levels of anandamide was observed. This UVB-induced decrease was positively correlated with the patients BMI.
9. Phototherapy for psoriasis failed to induce significant changes in LDL cholesterol, HDL cholesterol, triglycerides or glucose fasting levels and caused only a slight decrease in leptin in males. Although the recognized role of endocannabinoids on metabolic syndrome, no correlation was observed between endocannabinoid levels and the studied metabolic parameters.

Summary
Resumo

Summary

Ultraviolet radiation (UVR) is a permanent environmental threat, and the skin is endowed with several adaptive mechanisms against its damaging effects. In the present work a fully functional endocannabinoid system in cell lines of human keratinocytes and melanocytes and its response to ultraviolet B (UVB) radiation are described. These cutaneous cells express both CB₁ and CB₂ receptors, produce physiologically relevant concentrations of endocannabinoids and have the main cannabinoid metabolizing enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL).

We report that UVB radiation up-regulates this cutaneous endocannabinoid system. In keratinocytes, UVB increases the levels of anandamide, 2-arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA) and the activities of MAGL and FAAH. On the other hand, in melanocytes, UVB increases the mRNA expression of CB₁ receptors. In keratinocyte-melanocyte co-cultures, UVB activates a paracrine CB₁-mediated signaling that decreases melanin synthesis. Moreover, anandamide synthesized by keratinocytes after UVB irradiation acts at transient receptor potential vanilloid receptor type 1 (TRPV1) channel in an autocrine manner and reduces their survival. Our findings suggest that endocannabinoids have a role in the cross talk between keratinocytes and melanocytes, after exposure to UVR.

The enzyme catechol-*O*-methyltransferase (COMT) was investigated as another potential protective mechanism against UVB irradiation. We show that similarly to melanocytes, keratinocytes also have COMT activity and that UVB radiation decreases COMT activity in both cell types. COMT inhibition did not modify melanin levels but increases UVB-induced cell death in keratinocytes.

Psoriasis is the prototypic disease successfully treated with UVB. Moreover, psoriasis patients have an increased risk of obesity and metabolic syndrome that are well-known pathological conditions associated with endocannabinoid system dysregulation. Thus psoriasis patients were selected to study UVB effects *in vivo*.

In psoriasis patients, basal anandamide plasma levels were positively correlated with the levels of high sensitive C-reactive protein (hsCPR), a biomarker for cardiovascular risk and inflammation. After UVB treatment a significantly decrease in the plasma levels of anandamide was observed. Although endocannabinoids have a recognized role on metabolic syndrome, no correlation was observed between endocannabinoid levels and the metabolic parameters studied.

We also report that UVB significantly decreased S-COMT activity *in vivo* as it was observed in cutaneous cell lines. No correlation was observed between COMT activity and clinical response. Interestingly, these patients have significantly higher S-COMT activity than controls. In addition, we report that UVB significantly increases the vitamin A and vitamin D serum levels in psoriasis patients.

In conclusion, the results presented here show that UVB radiation modifies endocannabinoid system activity and that the cutaneous endocannabinoid system may participate in the UVB effect on melanogenesis and cell viability.

Resumo

A pele dispõe de vários mecanismos que a protegem da agressão pela radiação ultravioleta (UV).

Neste trabalho descreve-se um sistema endocanabinoide funcionalmente reativo à radiação ultravioleta B (UVB), em linhas celulares de queratinócitos e melanócitos humanos. Estas células cutâneas produzem concentrações fisiologicamente relevantes de endocanabinoides, exprimem os recetores dos canabinoides (CB₁ e CB₂) e têm as principais enzimas de degradação dos endocanabinoides, hidrolase das amidas dos ácidos gordos (FAAH) e a monoacilglicerol-lipase (MAGL).

Este sistema endocanabinoide cutâneo é ativado pela radiação UVB. Nos queratinócitos, a radiação UVB aumenta os níveis de anandamida, 2-araquidonoilglicerol (2-AG) e palmitoiletanolamida (PEA), e a atividade das enzimas MAGL e FAAH. Nos melanócitos, a radiação UVB aumenta a expressão do RNAm dos receptores CB₁. Em co-culturas de queratinócitos e melanócitos, a radiação UVB ativa uma inibição parácrina da síntese de melanina, mediada pelos recetores CB₁. A anandamida sintetizada pelos queratinócitos após a radiação UVB atua de forma autócrina nos canais de tipo vaniloide 1 do potencial transitório dos fotorrecetores (TRPV1) e aumenta a morte celular. Estes resultados mostram que os endocanabinoides têm um papel importante na interação entre queratinócitos e melanócitos, que se torna mais evidente após exposição à radiação UV.

A enzima catecol-*O*-metiltransferase (COMT) foi estudada como um possível mecanismo protetor da pele contra a radiação UVB. Verificou-se que tal como os melanócitos, também os queratinócitos têm atividade da enzima COMT e que esta é diminuída pela radiação UVB. A inibição da COMT não modifica os níveis de melanina, mas aumenta a morte celular induzida pela radiação UVB nos queratinócitos.

A radiação UVB é um dos tratamentos de primeira linha na psoríase. Por outro lado os doentes com psoríase têm um risco aumentado de obesidade e síndrome metabólico que são patologias reconhecidamente associadas à desregulação do sistema endocanabinoide. Assim, escolhemos os doentes com psoríase para estudar *in vivo* os efeitos da radiação UVB.

Em doentes com psoríase, observamos uma correlação positiva entre os níveis plasmáticos basais de anandamida e os níveis de proteína C reativa medidos pelo método de alta sensibilidade (hsCPR) que são um marcador de risco cardiovascular e inflamação. Após a fototerapia com UVB, os níveis plasmáticos de anandamida diminuíram significativamente. Apesar dos endocanabinoides terem um papel reconhecido na síndrome metabólica, não se observou qualquer correlação entre os níveis de endocanabinoides e os parâmetros metabólicos estudados.

Em concordância com os resultados em linhas celulares cutâneas verificou-se que a fototerapia UVB reduz significativamente a atividade da S-COMT eritrocitária em doentes com psoríase. Os doentes com psoríase têm maior atividade basal da COMT eritrocitária que os controlos mas não se observou correlação entre a atividade da COMT e a resposta clínica à fototerapia. Demonstramos também nestes doentes que a radiação UVB aumenta significativamente os níveis séricos de vitamina A e de vitamina D.

Em conclusão, os resultados aqui apresentados mostram que a radiação UVB altera a atividade do sistema endocanabinoide e que o sistema endocanabinoide cutâneo parece contribuir para os efeitos da radiação UVB na melanogénese e na viabilidade celular.

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