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PHARMACOLOGIC INDUCTION OF THE MELANOCOTIN 1 RECEPTOR (MC1R) PATHWAY PROVIDES PROTECTION AGAINST SUNBURN AND ENHANCES EXPRESSION OF ANTIOXIDANT ENZYMES IN THE SKIN

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PATHWAY PROVIDES PROTECTION AGAINST SUNBURN AND ENHANCES
EXPRESSION OF ANTIOXIDANT ENZYMES IN THE SKIN.

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy in the College of Medicine at the University of Kentucky

By

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2015

ABSTRACT OF DISSERTATION

PHARMACOLOGIC INDUCTION OF THE MELANOCORTIN 1 RECEPTOR (MC1R) PATHWAY PROVIDES PROTECTION AGAINST SUNBURN AND ENHANCES EXPRESSION OF ANTIOXIDANT ENZYMES IN THE SKIN.

The inability to tan properly after sun exposure strongly correlates with increased incidence of skin cancer. The melanocortin 1 receptor (MC1R) is a transmembrane Gs-coupled cell surface receptor found on epidermal melanocytes that transmits pro-survival and pro-differentiation signals mediated by the second messenger cAMP. Humans carrying loss-of-function polymorphisms in MC1R signaling exhibit higher incidences of skin cancers including melanoma.

This study focused on the physiologic effects of topical application of forskolin, an adenylate cyclase activator, in *extension* (Mc1r$^{e/e}$) *K14-SCF* animals, which model the fair-skinned UV-sensitive human. Twice daily application of the drug promoted accelerated pigmentation, increased skin darkening due to epidermal deposition of melanin pigment, and induced epidermal melanin, which protected the skin against UV injury as judged by "minimal erythematous dose" (MED). Moreover, MC1R signaling regulated the expression of antioxidant enzymes at the transcriptional level. The human melanoma cell line A375, known to harbor a loss-of-function signaling mutation in MC1R, was used to determine effects of cAMP stimulation on the expression of antioxidant enzymes. We observed increases in expression of genes that control the biosynthesis and regulation of glutathione including the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), glutathione peroxidase, GPX, and glutathione reductase GSR. In addition, there is an increase in manganese superoxide dismutase (MnSOD) at the protein level. There was accumulation of MnSOD in the mitochondria after pharmacologic induction of cAMP with forskolin. Addition of the oxidative agent H_2O_2 enhanced the expression of MnSOD at the protein level as early as one hour after MC1R stimulation. Oxygen consumption rate on mitochondria was measured using Seahorse analysis; pharmacologic activation of MC1R/cAMP signaling did not affect mitochondrial metabolism. In addition, topical application of a crude extract of *Solidago* inhibited UV-induced inflammation in *K14-SCF* mice. Several UV-induced cytokines, including TNF-α, were down-regulated at the transcriptional level after topical application of Solidago extract.

Together, these results indicate that MC1R signaling protects melanocytes from UV damage by regulating antioxidant enzyme expression and suggest that pharmacologic cAMP induction may be a useful preventive mechanism against UV-mediated skin sunburn and oxidative injury.

KEYWORDS: UV, skin cancer, melanocortin 1 receptor (Mc1r), forskolin, cAMP.

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Alexandra Amaro-Ortiz
Student's Signature

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Date

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Dedicated to my family and God

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CHAPTER 1

INTRODUCTION

THE SKIN

The skin is the largest organ of the human body with an average area of 1.5 square meters

and it consists of 16% of total body mass. The skin provides physical protection to the

body from environmental, chemical and microbiologic treats. The skin thickness varies

around the body with a range of 1.0 mm in the eyelids and 4 mm in the ball of the foot

(Stucker, Struk et al. 2002). Epithelial, mesenchymal, granular, and neurovascular

components come together to compose the skin. Once it is fully developed, there are two

main layers: the epidermis and the dermis.

THE EPIDERMIS

The epidermis consists of mainly keratinocytes and it is the outmost layer of the skin. The

epidermis can be as thin as 0.1mm and it is characterized by several functional layers of

differentiated keratinocytes (Fig. 1-1). The epidermis is a self-renewing tissue; there is

constant division of keratinocyte stem cells in the stratum basale to move from the basal

layer to form nascent epidermal keratinocytes. These cells accumulate keratin, melanin

pigment and form tight junctions with each other. The outermost layer of the epidermis

consists of terminal differentiated corneocytes that are dead, yet intact cells that are

essential to create the protective physiochemical barrier of the epidermis through tight-

linkages (Goodman, Miller et al. 2002).

MELANOCYTES

The skin is organized into "epidermal melanin units" wherein many keratinocytes interact

with one melanocyte (Fig 1-1). Melanocytes are the second most common cell type in the

epidermis (Barker, Dixon et al. 1995). Melanocytes are derived from neural crest cells that

have migratory characteristics together with peripheral and enteric neurons that migrate to specific locations during embryonic development. These cells are called melanoblast cells, which are un-pigmented and undifferentiated precursors of melanocytes that commit a dorsal-ventral migration to its final location in the body. Melanoblast differentiate to melanocytes once established in the basal layer of the epidermis. However, melanocytes are also found in the inner ear, the iris and behind the retina. Once located in the skin, melanocytes synthetize melanin in melanosomes. The melanosomes are vesicles responsible for the storage and transport of melanin to keratinocytes upon exposure to solar radiation. Keratinocytes receive melanin pigments from melanosomes (Easty, Patel et al. 1988, Lowings, Yavuzer et al. 1992) providing a barrier against accumulation of damaging levels UV radiation.

THE DERMIS

The dermis is located directly below the epidermis and it can be as thick as 2mm. The epidermis and the dermis are separated by a thin basement membrane. The dermis is a supportive layer of the skin with most dermal cells being derived from mesoderm. The most common feature of the dermis is the presence of hair follicles among other components of cutaneous structures including nerves, blood vessels, sebaceous glands and sweat glands. The presence of immune cells in the dermis contributes to the immune responses, and fibroblast cells participate in the formation of the extracellular matrix and collagen.
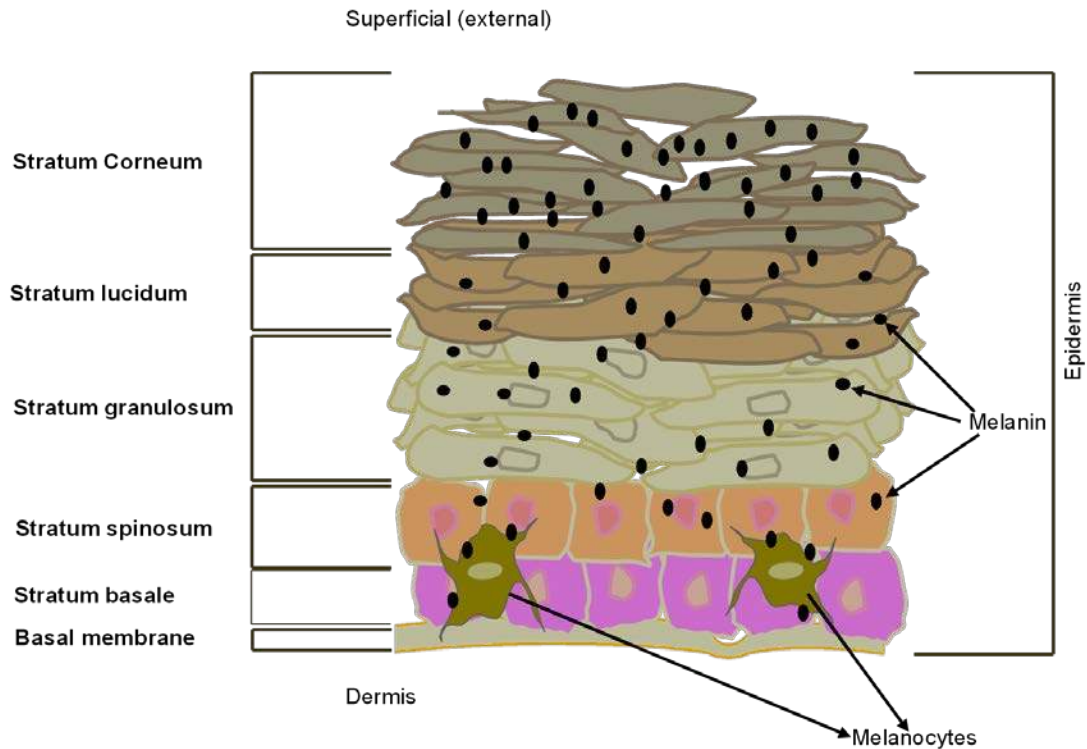
Figure 1-1. Schematic representation of epidermal differentiation.

The epidermal unit shows the presence of melanocytes at the basal membrane. The keratinocytes are located around the melanocytes and start to differentiate from the stratum basale. There are three differentiation stages stratum spinosum, granulosum and lucidum. Each layer is characterized by the induction and accumulation of different skin markers such as keratins, cornifin, filaggrin among others while the cells are differentiated to the surface. The corneum layer is composed of dead that serve as the outer layer of the skin.

ULTRAVIOLET (UV) AND SOLAR RADIATION

Due to its anatomic location at the external boundary of the body, the skin is exposed to a variety of environmental factors such as UV radiation that derives naturally from the sun. UV radiation is composed of UVA, UVB and UVC components based on photon wavelength. UVA has the longest wavelengths (315-400 nm), UVB mid-range in the spectrum (290-320 nm) and UVC has the shortest wavelengths (100-280 nm). Ambient sunlight exposure is composed primarily of UVA (90-95%) and UVB (5-10%) energy, with most solar UVC absorbed by the ozone layer (Fig. 1-2). UV radiation is an environmental factor that induces skin pathologies, including erythema (Wenczl, Pool et al. 1997, Amaro-Ortiz, Vanover et al. 2013, D'Orazio, Jarrett et al. 2013), inflammation (Rees 2004), aging effects (Dalziel 1991), sunburn (Autier and Dore 1998, Mallet, Kypriotou et al. 2013) and cancer. Unprotected exposure to solar radiation is very common and this leads to deleterious cutaneous effects.

UVA and UVB spectra penetrate the skin differently. Variations in skin thickness and the presence of melanin influence absorption of UV light. Epidermal melanin is able to transmit and reflect UV emissions of less than 300 nm (Anderson and Parrish 1981) which explains why UVA penetrates the skin more deeply than UVB despite UVA having a lower energy profile to UVB. Similarly to melanin, other molecules like urocanic acid and the amino acids tryptophan and tyrosine can absorb lower UV wavelength. On the other hand, the dermis seems to have more transmittance and reflectance for longer wavelength such as the one emitted by UVA. Dermal scattering is higher at lower wavelength allowing UVA-visible-infrared spectrum wavelengths to penetrate the dermis (Anderson and Parrish 1981). Other components of the dermis such as hemoglobin, bilirubin and beta-carotene are major absorbents of visible radiation compared to melanin in which the absorption of visible spectrum is negligible (Anderson and Parrish 1981, Parrish, Zaynoun et al. 1981).
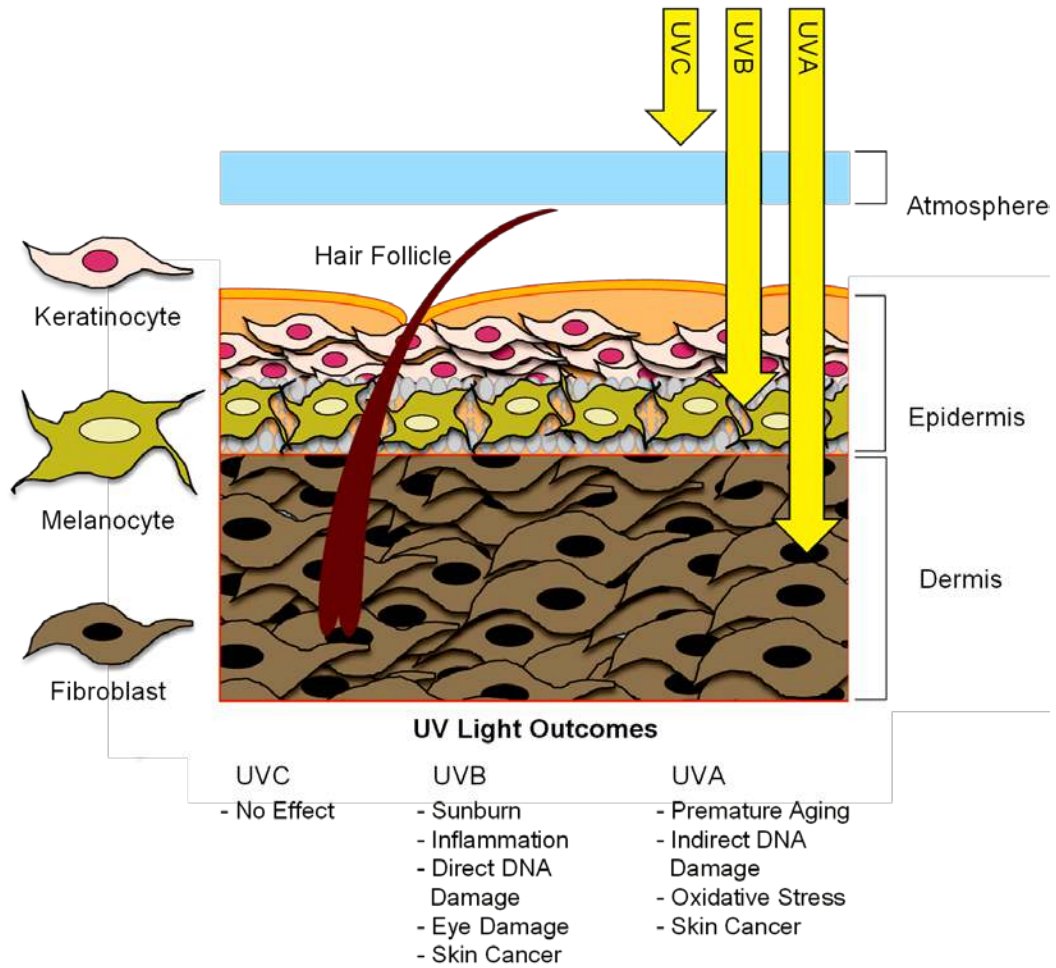
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Figure 1-2. UV exposure of the skin.

UV radiation in ambient sunlight is composed primarily of UVA and UVB energy. The ozone absorbs most UVC; therefore, although it is highly bioactive, terrestrial organisms are not exposed to significant levels of UVC. UVB can cause direct damage to DNA and reach the epidermis. UVA can penetrate the dermis and increases levels of ROS that indirectly induce DNA mutagenesis. The outcomes of both, UVA and UVB, in the skin are listed.

UVB is a well-characterized mutagen and inducer of skin cancers (Barker, Dixon et al. 1995, Darwiche, Bazzi et al. 2005, Ahsan, Reagan-Shaw et al. 2007, Grant 2008), but recent studies have implicated an increasing role for UVA as a carcinogen (Paunel, Dejam et al. 2005, Venditti, Bruge et al. 2011, Akhalaya, Maksimov et al. 2014) likely through its pro-oxidative effects and possibly through other mechanisms such as telomere shortening (Yin and Jiang 2013). In addition, UVA is less able to induce melanin production compared to UVB, leaving the skin less able to protect itself against further UV insult (Atillasoy, Seykora et al. 1998, Grant 2008, Wendt, Schanab et al. 2012, Flament, Bazin et al. 2013, Yin and Jiang 2013). Increasing attention is being paid to the potential impact of UVA radiation to areas of the body rarely exposed to natural UV, including the vulva and oral mucosa, even focusing on differential cellular repair and apoptosis depending on anatomic site (Breger, Baeva et al. 2013). Much of solar UV energy is absorbed by stratospheric ozone, and the gradual depletion of stratospheric ozone over the last several decades has resulted in higher levels of solar UV radiation that strikes the surface of the Earth (Norval, Lucas et al. 2011). Increased ambient UV radiation from global climate change may be an important factor to explain the increasing prevalence of melanoma and skin cancer over the last several decades (Armstrong and Kricker 2001, Diffey 2004, Garbe and Leiter 2009, Rigel, Russak et al. 2010). Both direct and indirect DNA changes interfere with transcription and replication, and render skin cells susceptible to mutagenesis. UV radiation induces a variety of free radical and oxidative molecules, which because of their chemical reactivity alter the molecular structure and damage lipids, proteins and nucleic acids (Chedekel and Zeise 1988).

UV-INDUCED DIRECT DNA DAMAGE

UV light has direct effects on DNA bases. The bases most susceptible to UV injury are pyrimidines. The rates of UV signature lesions include two third cyclobutane pyrimidine

dimers (CPD) and one third 6-4 pyrimidine photoproducts (6-4PP) caused by UV (Roza, van der Wulp et al. 1988, Estil, Olsen et al. 1997). CPD are formed when there is a base substitution of a cytosine → thymine at a di-pyrimidine site. On the other hand, 6-4PP are formed when two adjacent pyrimidines undergo photochemical reaction at the 6th and 4th position of the pyrimidine residues forming a novel 5-6 double bond with the exocyclic moiety of an adjacent 3' pyrimidine (Johns and Butler 1964, Sarasin 1999). The shift of the helical distortion in the backbone of DNA caused by 6-4PP blocks DNA replication. Both of these lesions distort the double helix and can lead to mutation. An individual skin cell may accumulate up to 100,000 such lesions from one day's worth of sun exposure (Hoeijmakers 2009). These UV lesions if not repaired can lead mutagenesis. There are mechanisms of DNA repair available for the removal of these lesions.

MECHANISM OF DNA DAMAGE REPAIR

The nucleotide excision repair (NER) pathway is the main mechanism for the repair of CPD and 6-4PP. The NER pathway involves two types of repair, Transcription Coupled (TCR) and Global Genome Repair (GCR) (Gillet and Scharer 2006) that only differ in their damage recognition complex. Deficiency in any of the enzymes involved in NER leads to Xeroderma Pigmentosum (XP), a clinical condition characterized by chronic and excessive UV sensitivity. Many of the enzymes involved in this pathway have been named after this condition. TCR involves RNA polymerase which stalls at a lesion in the DNA template of actively transcribed genes. GGR is not dependent on transcription but senses damage by the XPC-HHR23B complex that recognizes distortions in the DNA helix. After damage is found, there is recruitment of other NER factors like XPE. XPA unwinds the DNA followed by RPA that stabilized the DNA for the recruitment of XPB and XPD. XPG and XPF promote excision of the damaged oligonucleotide and DNA polymerase promotes DNA synthesis with a DNA ligase finalizing the repair process.

The NER pathway may fail to repair UV-damage DNA. In those cases, the translesion DNA synthesis (TLS) pathway is the cellular mechanism (DNA damage tolerance) in response to DNA lesions caused during replication. This pathway includes three polymerases η, ζ and REV1 that repairs DNA in an error-free (mutation avoiding) or error-prone (mutation generating) mechanism (Goodman, Miller et al. 2002, Tsaalbi-Shtylik, Verspuy et al. 2009). During replication via an error-free mechanism, pol η uses the un-damaged strand as a template to prevent mutation while REV1 creates a deletion by by-passing the damaged region during replication (Tsaalbi-Shtylik, Verspuy et al. 2009). Other types of UV lesions are found on C-phosphate-G (CpG) sites such as methylation of cytosine, 5mC (Ikehata, Kumagai et al. 2013). This mutation is common in the tumor suppressor gene p53 (van Kranen, de Gruijl et al. 1995, Cui, Widlund et al. 2007).

UV-INDUCED INDIRECT DNA DAMAGE

UV radiation also damages cellular macromolecules indirectly, through production of oxidative free radicals (Meyskens, Farmer et al. 2001). Several DNA modifications can result from oxidative injury, including 7, 8-dihydro-8-oxoguanine (8-OHdG), which promotes mutagenesis (specifically GC-TA transversion mutations) (Schulz, Mahler et al. 2000). UVA-induced ROS in the skin causes oxidation at the eighth position of guanine producing 8-OhdG (Nishimura 2002, Kunisada, Sakumi et al. 2005). This DNA product, if not repaired, will pair with an adenine instead of cytosine. Then, in the next step of replication, the adenine will pair with a thymine inducing a mutation. Cellular maintenance pathways exist to inactivate oxidative species as well as to repair the DNA damage these species may cause.

MECHANISM OF DNA DAMAGE REPAIR

The base excision repair pathway (BER) is the main molecular mechanism by which cells reverse free radical damage in DNA to avoid oxidative mutagenesis (Wyatt, Allan et al.

1999, Liu, Prasad et al. 2007). This pathway is initiated by lesion-specific glycosylases that scan DNA for specific alterations including deaminated, alkylated or oxidized bases. The glycosylase known to remove 8-OhdG is called 8-oxoguanine glycosylase, OGG. OGG has been studied as a key DNA repair enzyme after UV- induced DNA oxidation (Kadekaro, Chen et al. 2012). The glycosylase cleaves the N-glycosidic bond that connects the base to the sugar, creating an abasic site (AP site). The generation of the AP site recruits an AP endonuclease to hydrolyze the phosphodiester bond while the glycosylase cleave the 3' AP-Site. The residues that are formed are processed by deoxyribose phosphodiesterase that removes the residual deoxyribose phosphate unit to facilitate DNA synthesis with a DNA polymerase and a DNA ligase seals the strand (Wyatt, Allan et al. 1999).

MELANIN

UV radiation can directly damage the skin. Melanin deposited in keratinocytes can efficiently block the penetration of UV radiation in the skin (Hollis and Scheibner 1988). A critical mediator of UV sensitivity and skin cancer risk is skin complexion, which is determined primary by the amount and type of melanin present in the epidermis. Melanin is derived from the amino acid tyrosine. Melanin is a large bio-aggregate of pigmented chemical species (Fig. 1-3). Tyrosine is converted through various steps to melanin (Jimbow, Alena et al. 1992, Fuller, Drake et al. 2000). Tyrosinase is the rate-limiting biosynthetic enzyme in the melanogenesis pathway, as it catalyzes the first two steps of melanin synthesis (conversion of tyrosine to DOPA and then to DOPAquinone). There are two major "mature" forms of melanin known as eumelanin and pheomelanin (Wakamatsu, Kavanagh et al. 2006, Ito and Wakamatsu 2011). The synthesis of melanin occurs by successive oxidation and cyclization steps (Kim, Song et al. 2010, Kim 2014, Kim, Baek et al. 2015).

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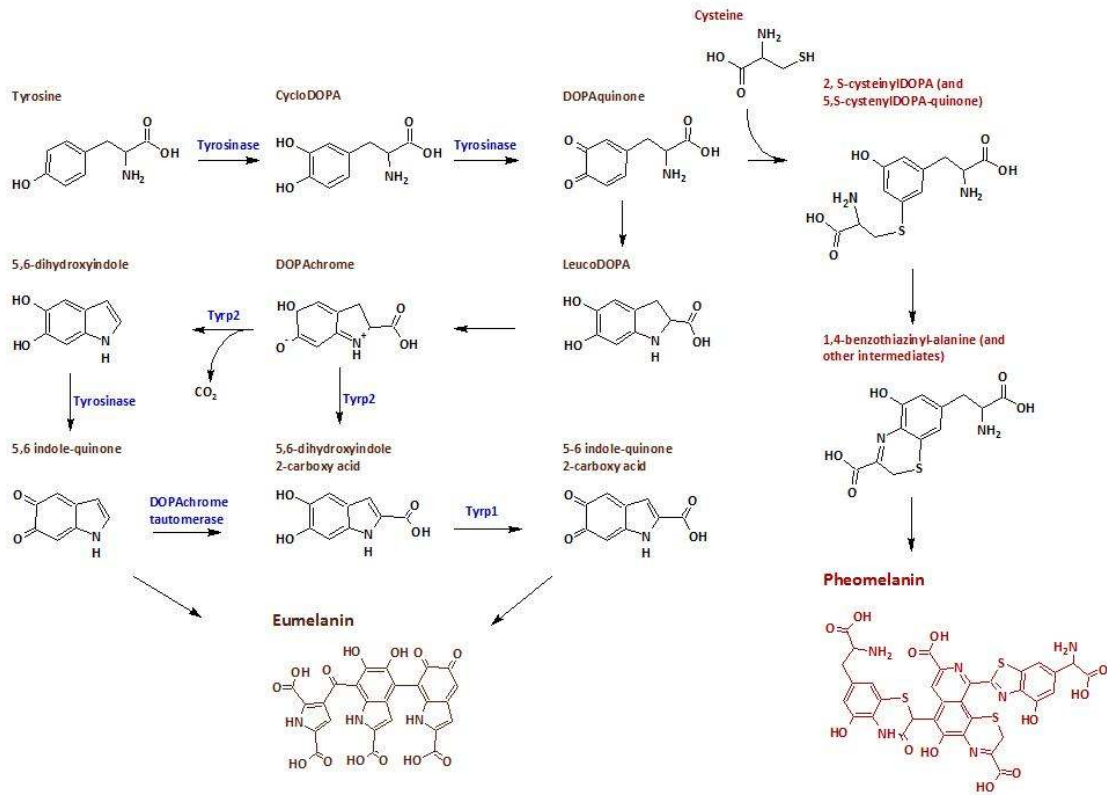
Figure 1-3. The synthesis of melanin.

There are two forms of melanin: dark/black eumelanin and light/red pheomelanin. Both eumelanin and pheumelanin are derived from the amino acid tyrosine. Tyrosinase catalyzes the rate limiting reaction from the synthesis of melanin. The incorporation of cysteine in the biosynthesis of melanin leads to the light/red color of pheomelanin. There are other enzymes that trigger the synthesis of dark/black eumelanin (Tyrp1, Tyrp2, tautomerase). The expression of mutated Tyrosinase has a phenotype of albinism.

Eumelanin protects against UV exposure by interfering with UV entry into the deeper, sensitive layers of the skin. Eumelanin is a dark brown/black pigment in contrast to pheomelanin, which is a light red pigment less able to block UV. Pheomelanin is a sulfur containing pigment that may actually potentiates UV injury in the skin. Pheomelanin contributes to free radical formation and oxidative cellular damage (Mitra, Luo et al. 2012). In addition, the structure of pheomelanin is photosensitive and increases pro-oxidant levels in the skin (Salopek, Yamada et al. 1991). Therefore, individuals with high levels of pheomelanin and low levels of eumelanin in the skin are UV sensitive and are at a greater risk for developing skin cancers including melanoma (Wakamatsu, Kavanagh et al. 2006). If the skin is continually stimulated for melanin production, the melanin will stay and deposit in keratinocytes until the UV exposure is removed. Dark pigmented skin is protected against formation of CPD (Yamaguchi, Coelho et al. 2008), photoproducts (de Lima-Bessa, Armelini et al. 2008), and a decrease in oxidative stress (Jenkins and Grossman 2013).

Various inhered and environmental factors control the ratio of eumelanin-to-pheomelanin in total amount of melanin. When melanocytic cytoplasmic cAMP levels are high, eumelanin production is favored compared to lower levels of cAMP that induce the synthesis of pheomelanin. Individuals can be categorized by phenotype of skin complexion using the Fitzpatrick Scale. This scale ranges from very fair skin (e. g. Northern Europeans type I) to very dark (e. g. for Aboriginal African type VI). Risk of sunburn is linked to not only UV dose and intensity of UV exposure, but also by hereditary factors that influence cutaneous response to UV radiation. Many factors determine melanocytic response to UV. Skin cancer risk is inversely proportional to phenotype scale in which a type 1 skin complexion has a higher risk than type VI.

SKIN CANCER

Skin cancer is the most diagnosed cancer in United States and it is caused by an uncontrolled growth of genetically mutated skin cells. There are keratinocyte and melanocyte malignancies that, together, are by far the most numerous cancers diagnosed today (Lo and Fisher 2014). Skin pigmentation is one of the most important determinants of UV sensitivity. Melanoma, the most deadly form of skin cancer, occurs roughly twenty times more frequently in light-skinned persons compared to their dark-skinned (Tucker 2009, Psaty, Scope et al. 2010, Udayakumar, Mahato et al. 2010). Because of low innate levels of eumelanin pigment in the epidermis, fair-skinned individuals are much more prone to acute and chronic effects of UV radiation including sunburns, photo-aging and skin cancers (Conley and Pack 1963, Evans, Kopf et al. 1988, Gibson, Donald et al. 1997). Besides innate skin pigmentation, the ability to tan after sun exposure is also another important determinant of UV sensitivity (Suzuki, Im et al. 1999, Abdel-Malek, Knittel et al. 2008, Munoz-Hidalgo, Lopez-Gines et al. 2014).

NON MELANONOMA SKIN CANCER

Non-melanoma skin cancer (NMSC) develops from abnormal keratinocytes. These types of cancer include basal cell (BCC) or squamous cell carcinomas (SCC), which are generally diagnosed early and are treated surgically. Actinic keratosis (AK) was identified as an early stage of NMSC and if left untreated, up to ten percent of cases of AK can develop into SCC. There is an estimate increase of 200 percent in the diagnosis of SCC in USA since 1990. This type of cancer is generated in outer layers of the epidermis, starting in a localized area in the skin and caused by long-term exposure to solar radiation. BCC accounts for 80% of NMSC cases and almost never metastasizes (Gupta, Daigle et al. 2014). BCC is generated from deeper areas of the epidermis and has been characterized for its slow growth. BCC is caused after long-term exposure to the sun or

occasional intense episodes that lead to sunburn. There are different therapeutic options after being diagnosed with NMSC. Electro-desiccation of a tumor has cure rates of 95%. Other procedures include tissue removal by surgery, cryosurgery, laser surgery, topical medication, radiation, and among others.

MELANOMA

Melanoma accounts for almost 10,000 deaths each year in the US alone, despite representing a small fraction of the total number of skin malignancies (Bristow, Casil et al. 2013). Melanoma, derived from melanocytes, is usually curable at early stages. However, once it advances and invades other tissues, it is generally resistant to cancer treatments. It is prone to spreading throughout the body from its site of origin (most frequent in the epidermis). For a variety of reasons, the prevalence of melanoma has been steadily increasing in Western countries for decades. Whereas only one in approximately 1,500 Americans were diagnosed with melanoma in his/her lifetime in the 1930's, today that number has increased to roughly one in sixty people. Risk factors include family history of skin cancer, weakened immune response, moles, and sun exposure. There are two types of moles: benign moles and atypical moles, also known as dysplastic nevi. Even without family history, individuals with a high number of moles face a high risk of developing melanoma.

MELANOMA PROGRESSION

Most melanomas are derived from moles (nevi). At the molecular level, dysplastic nevi have an 80% frequency in mutations of BRAF, an oncogene that controls melanocytes cell growth (Fig. 1-4). Specifically, *BRAF*^{V600E} leads to an increase in cell proliferation followed by cell senescence triggered by CDK4 activation, a regulator of cell cycle (Bertolotto 2013, Munoz-Hidalgo, Lopez-Gines et al. 2014). CDK4 loss leads to a radial phase growth in the progression of melanoma and, PTEN mutation leads to the activation
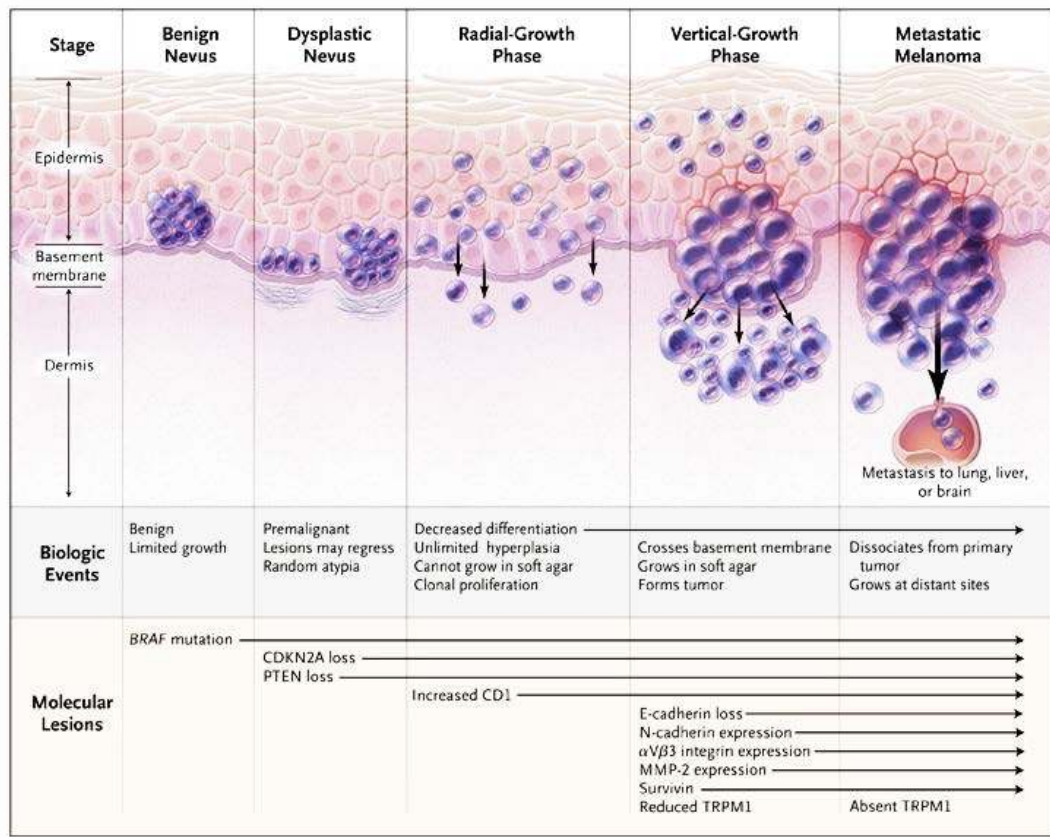
Figure 1-4. The progression of skin cancer.

Benign nevi show a BRAF mutation and activation of the MAPK signaling pathway. Atypical dysplastic nevi show loss of cyclin dependent kinase inhibitor (CDKN2A) and PTEN leading to an impaired cell cycle control. The growth phase of melanoma is characterized by up-regulation of cell adhesion signaling pathway. Other genes that loss regulated signaling are E-cadherin, N-cadherin, MMP-2 and TRPM1 a melanocyte-specific gene melastatin 1 (Miller and Mihm 2006).

of PI3K-AKT pathway that favors early stages of invasive melanoma. The activation of E-cadherin and WNT5A lead to the invasion into the dermis, the vertical growth phase. At this point, the aggressiveness of the cells lead to metastatic effects due to the loss of regulation of cellular proliferation, cell survival and apoptosis pathways.

Current therapeutic treatment for patients with melanoma is an initial procedure involving the surgical removal of the localized tumors. However, for specific cases, a physician might recommend a round of chemotherapeutic drugs targeting different oncogenes such as BRAF and MEK. The use of vemurafenib (Grimaldi, Cassidy et al. 2014), a BRAF inhibitor and trametinib a MEK inhibitor (Yajima, Kumasaka et al. 2012) are increasingly used in treatment of melanoma. Usually, these treatments are recommended for advance stages of melanoma leading to a better prognosis for survival. However, only a percentage of melanoma cases have mutations in BRAF, restricting therapeutic options for patients.

Melanoma occurs roughly twenty times more frequently in light-skinned persons compared to dark-skinned individuals (Aubin, Humbey et al. 2001). The incidence of melanoma in the United States has increased dramatically over the last several decades, particularly among fair-skinned individuals. Strong molecular and epidemiologic evidence supports the hypothesis that UV radiation is a major mutagenic environmental carcinogen responsible for melanoma (Gallagher, McLean et al. 1990, Kraemer, Lee et al. 1994, Wang, Yu et al. 2009, Pleasance, Cheetham et al. 2010, Parkin, Mesher et al. 2011, Wang, Smith et al. 2014). The role of UVA in melanoma formation is suggested by the observation of rising melanoma incidence over the last several decades and sunscreen use in the 1980s when only UVB-blocking sunscreens were used. UV exposure is thought to be a causal factor in the majority of melanoma cases. Clearly the more UV a person is exposed to (particularly strong intermittent doses such as those that cause sunburns), the higher his/her risk of melanoma.

Melanoma risk can be determine by the amount of sunlight exposure, occupational and recreational outdoor activities, amount of clothing/sun protection worn as well as proximity to the equator and altitude. However, a contributor factor for melanoma of increasing importance is the use of artificial UV (Dore and Chignol 2012). Proliferation of tanning beds use has played a role in this increase (Fisher and James 2010, Weinstock and Fisher 2010). There are more than one million people in United States of America using tanning beds per day (Spencer and Amonette 1998, Grimaldi, Cassidy et al. 2014). Several studies have estimated that indoor tanning bed use before the age of thirty-five, for example, increases lifetime risk of melanoma by as much as 75% (Schulman and Fisher 2009, Karagas, Zens et al. 2014). Even more than chronic lifetime UV exposure, melanoma risk seems particularly linked with sunburns (Pfahlberg, Kolmel et al. 2001), especially those early in life (Lew, Sober et al. 1983, Autier, Dore et al. 1998).

OXIDATIVE INJURY

The role of oxidative injury in the skin has been studied (Bickers and Athar 2006, Svobodova, Zdarilova et al. 2007, Piao, Ahn et al. 2014). Long exposure to UV radiation increases risk of keratinocytes malignancies (Potter, Gohde et al. 2000). Consequently, UVA has been found to have a role in the increased production of ROS (reactive oxygen species) in the skin. (Nataraj, Black et al. 1996) ROS are oxygen-like radical anions and molecules that due to their unstable electrons are very reactive. ROS are identified as superoxide radical O_2^-, hydroxyl radical OH^- and hydrogen peroxide, H_2O_2. Similar to ROS, reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($OONO^-$) increase the generation of oxidative stress. Oxidative stress is an imbalance in the generation of ROS and the antioxidant enzymes in the cells. Figure 1-5 shows a simplified scheme of the location of these species in the cell. Antioxidant enzymes are the main
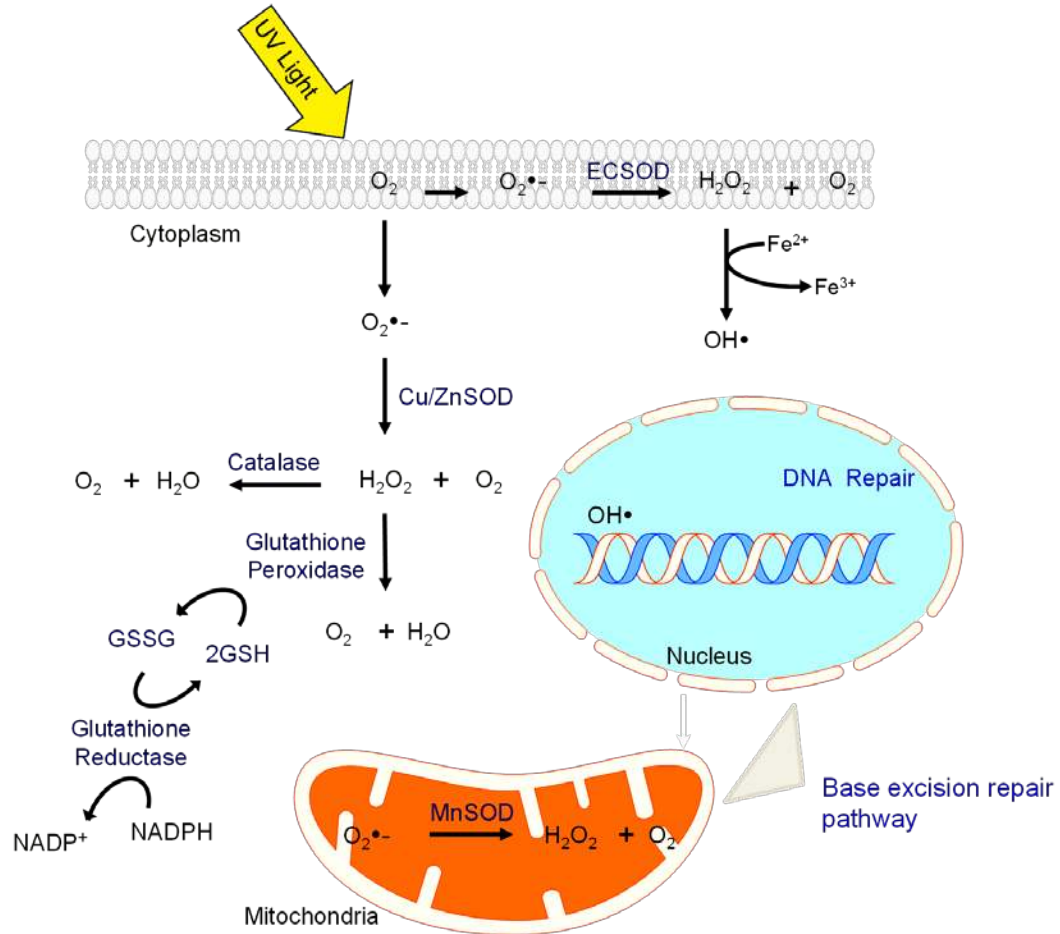
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Figure 1-5. Antioxidant enzymes and oxidative stress.

UV induces a variety of free radical and oxidative molecules, which because of their chemical reactivity damage lipids, proteins and nucleic acids. Antioxidant enzymes mediate the removal of ROS, with different enzymes functioning in specific compartments (e.g. MnSOD localized to mitochondria). If not removed, ROS may react with DNA and other cell signal proteins, impairing their function. GSH, Glutathione. ECSOD, Extracellular Superoxide dismutase. Cu/Zn SOD, copper/zinc superoxide dismutase. MnSOD, Manganese Superoxide dismutase.

regulators of the levels of ROS, acting to minimize ROS-mediated formation of DNA and proteins adducts and impairment of cellular function.

ROS are produced by cells during normal metabolic activities such as mitochondrial oxidative phosphorylation. Without inactivation, ROS can damage macromolecules including lipids, proteins and DNA. UV, particularly longer-wavelength UVA, is a well-known inducer of ROS, and UV-induced oxidative stress may be an important contributive factor for melanoma (Bossi, Gartsbein et al. 2008, Afanas'ev 2010, Choi, Uehara et al. 2012). ROS can inappropriately activate signaling pathways, interfere with genome maintenance, and affect apoptosis. Numerous studies have tested the effects of solar radiation and oxidative stress on the skin (Hu 2005, Poljsak and Dahmane 2012, Gabe, Osanai et al. 2014), and oxidative stress has been linked to age-related loss of skin elasticity (Langton, Sherratt et al. 2010, Mahmood, Akhtar et al. 2011, Naylor, Watson et al. 2011), defective cellular signaling (Prunier, Masson-Genteuil et al. 2012) and photo-aging (Stohs 1995, Lee, Cho et al. 2012).

ANTIOXIDANT ENZYME EXPRESSION

The term oxidative stress combines the excess of ROS with lower levels of antioxidant enzymes. The major antioxidant in most types of cell is glutathione (GSH). The role of GSH is to facilitate the depletion of H_2O_2 to H_2O. GSH is oxidized by glutathione peroxidase, GPX. GPX takes GSH and converts it to GSSG in order to reduce H_2O_2 to H_2O. Then, GSSG is recycled by glutathione reductase, GSR. GSR together with the co-factor NADPH produces GSH and $NAD(P)^+$. Another antioxidant enzyme that controls the levels of H_2O_2 is catalase. Catalase is a nuclear-encoded protein that is located in peroxisomes and its levels increase after UV exposure (Schallreuter, Moore et al. 1999) in melanocytes (Song, Mosby et al. 2009, Kadekaro, Chen et al. 2012). When there is depletion of GSH due to an increase in the levels of ROS, there is activation of the

transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Fig. 1-6) (Kokot, Metze et al. 2009). Nrf2 regulates the expression of glutamate cysteine ligase, GCLC, the enzyme that controls the catalytic limiting step for the synthesis of GSH. GCLC is known as γ-glutamyl cysteine synthase (Gipp, Chang et al. 1992). Therefore, there is recovery in the expression of GSH in a functional cell. Also, Nrf2 is involved in the regulation of GSR and glutathione-S-transferase, GSTA. GSTA is involved in the reduction of ROS-induced oxidized proteins (Kokot, Metze et al. 2009). The concentration of GSH decreases with skin tumor progression (Engin 1976, Bickers and Athar 2006). Levels of SOD are decreased in basal cell carcinoma (Nakagami, Inamura et al. 1991). As expected, several studies have found that there is an increase in the oxidative state of skin cancer cell lines (Engin 1976, Nakagami, Inamura et al. 1991, Liebler and Burr 2000, Schallreuter and Wood 2001). Taken together, the regulation of oxidative stress is the key to decreasing the burden of UVA-induced oxidative stress.

SUPEROXIDE DISMUTASE

Superoxide radical (O_2^-) is generated by photoexcitation of triplet oxygen $\left({}^3O_2 \right)$ that by transferring an electron to an intermediate singlet oxygen specie $\left({}^1O_2 \right)$ induces the formation of O_2^- (Mayeda and Bard 1974). Specific antioxidant enzymes that decrease levels of O_2^- radicals are called superoxide dismutases (SOD). They are found among different locations in the cells but their common role is the removal of superoxide radicals from the cell. Cu/ZnSOD is found in the cytoplasm and mitochondrial membrane. ECSOD is found anchored in the plasma membrane and its role is to regulate O_2^- radicals produced during lipid peroxidation such as 4-hydroxy-nonenal and malondialdehyde. In addition, there is endogenous production of O_2^- by the electron transport chain in the mitochondria. Levels of O_2^- in the mitochondria are depleted by MnSOD converting O_2^- to H_2O_2 for further conversion to H_2O by GPX or catalase. Polymorphisms in SODs are associated with
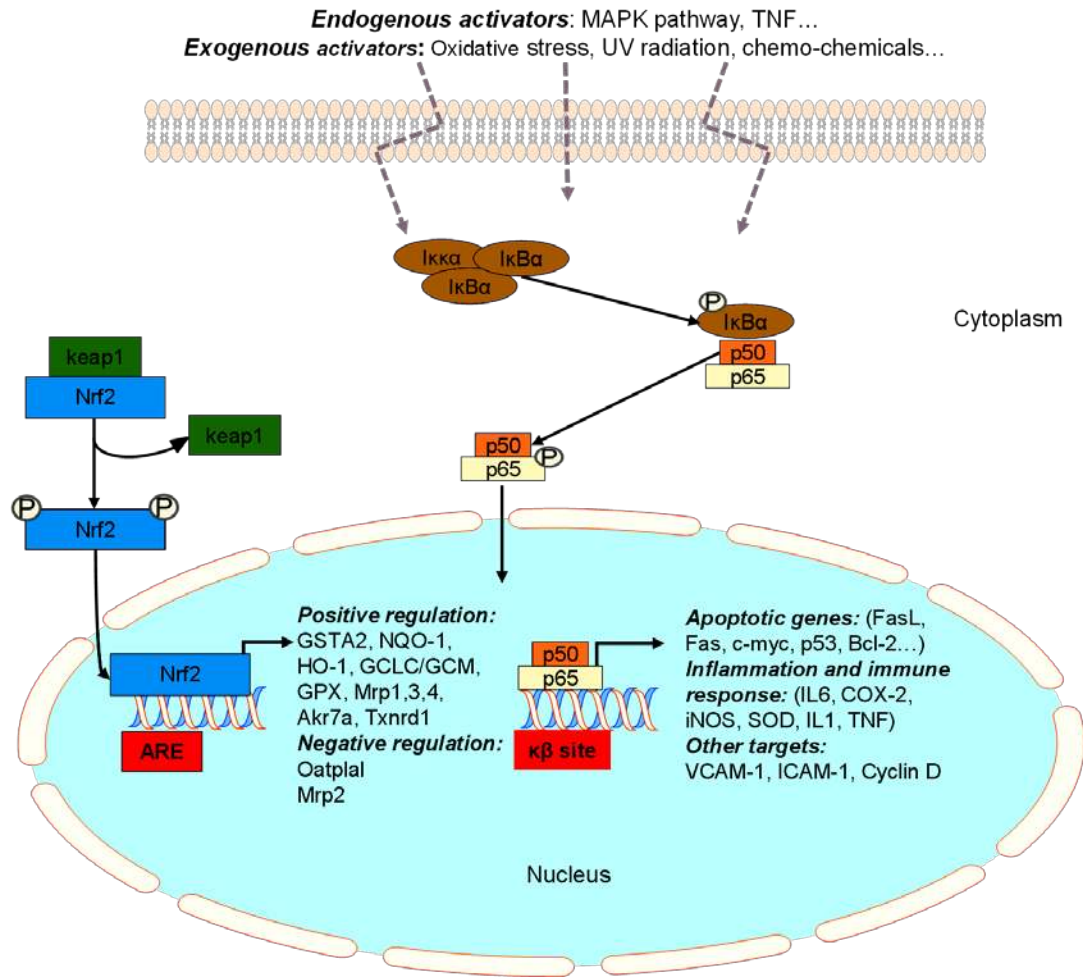
Figure 1-6. The signaling pathway of Nrf2 and NfκB after cell injury.

Endogenous and exogenous activators induce the release of Nrf2 from its inhibitor keap1 leading to keap1 degradation. Nrf2 translocates to the nucleus and binds to antioxidant response element (ARE) promoting the expression of different genes. Some of these genes are regulators of glutathione synthesis, the transporters MRP 1, 3, & 4 among other antioxidant genes. On the other hand, IκBα inhibits NF-κB in the cytoplasm. The phosphorylation of IκBα leads to its degradation and further phosphorylation of NF-κB. Nf-κB translocates to the nucleus binding to the κB site in DNA promoting the regulation of apoptotic genes, cell cycle regulators inflammatory signaling and immune responses.

cardiovascular and neurodegenerative disorders (Berneburg, Gremmel et al. 2005, Boutte, Woltjer et al. 2006, Zhang, Yang et al. 2009, Lai, Xu et al. 2013). Cu/ZnSOD and ECSOD are inhibited by H_2O_2; MnSOD is inactivated by peroxynitrite. The gene expression of SOD is regulated by nuclear factor kappa B, NF-κB (Dhar, Xu et al. 2010) (Fig. 1-6). NF-κB is inhibited in the cytoplasm by inhibitor kappa beta, IKβ (Dhar and St Clair 2012). NF-κB is activated by UV-mediated MAP Kinase pathway that phosphorylates IKβ. This inhibits IKβ and releases NF-κB to translocate to the nucleus and together with a co-regulator, activates the expression of many gene-encoding receptors, cytokines and antioxidant enzymes such as SOD. IKβ inhibits NF-κB translocation to the nucleus. Interestingly, the receptor of 1α,25-Dihydroxyvitamin D3, the active form of vitamin D, interacts with IKβ inhibiting NF-κB activity (Chen, Zhang et al. 2013). UV induces the generation of 1α,25-Dihydroxyvitamin D3 and by activating its receptor, stabilized IKβ and induces an attenuation of NF-κB nuclear translocation. Nonetheless, there is positive feedback for the degradation of IKβ upon the presence of endogenous or exogenous cellular stress activators. The kinetics for expression of several antioxidant enzymes at the transcriptional level usually is within hours (Lundqvist, Yde et al. 2014).

SKIN AGING AND ANTIOXIDANT DEFENSES

Because it triggers cellular damage pathways, oxidative stress activates cellular senescence, which is thought to directly lead to photo-aging (Velarde, Flynn et al. 2012, Yun, Kwon et al. 2012, Sakura, Chiba et al. 2013). Cellular senescence is associated with a reduced capacity to divide and proliferate, sometimes in conjunction with the shortening of telomeres (Kashino, Kodama et al. 2003, Yokoo, Furumoto et al. 2004, Davis, Wyllie et al. 2007, Makrantonaki and Zouboulis 2007). Yokoo et al. found that exposing cells to a pro-oxidant agent (H_2O_2) impaired telomerase function which eventually resulted in telomere shortening, decreased proliferation, and cellular enlargement (Yokoo, Furumoto

et al. 2004). Wrinkling of the skin is one of the most overt signs of photo-aging, and UV exposure can induce wrinkling over time (Raschke, Koop et al. 2004, Cornacchione, Sadick et al. 2007, Felippi, Oliveira et al. 2012, Kong, Shi et al. 2013). Though the molecular mechanism(s) of wrinkling are likely to be complex, UV exposure may reduce elastic properties of the skin to alter the three-dimensional structure of elastic fibers (Imokawa 2009).

There is a lot of interest in the study of antioxidant enzyme depletion leading to skin aging. The constant exposure to UV, allows the skin to show early symptoms of aging such as wrinkles, dryness and stiffness. Several natural products may reduce UV-induced oxidative damage. One mechanism of action of these natural agents is in the activation of the ascorbic acid pathway and reducing the levels of lipid peroxidation (Villacorta, Zhang et al. 2007). Another mechanism of antioxidant control is the activation of the Nrf2 pathway that has been implicated as a beneficial effect of resveratrol (Soeur, Eilstein et al. 2015). The use of resveratrol in human keratinocyte induces accumulation of cellular glutathione. Similar studies in mice showed that the main component in licorice root, 18B-glycyrrhetinic acid, induces expression of SOD and GPX (Kong, Shi et al. 2013). The use of natural flavonoids showed a beneficial effect against UV-induced inflammation (Masnec, Kotrulja et al. 2010, Nichols and Katiyar 2010). For example, flavonoids such as solidago induced anti-inflammatory signaling in reticulo-endothelial systems (Apati, Houghton et al. 2006, Wu, Takahashi et al. 2007, Lutz, Kulshrestha et al. 2014).

MELANOCORTIN 1 RECEPTOR (MC1R)

The MC1R is a 7-transmembrane protein located in the plasma membrane of melanocytes. MC1R is located on chromosome 16q24.3 in human and in mouse chromosome 8: 123,407,107-123,410,744. It is a 34.7 kilodalton transmembrane Gs-coupled hormonal receptor (Smith, Box et al. 2001). The role of MC1R is to control the

ratio of eumelanin and pheomelanin synthetized in the skin and hair of many species. In mice, MC1R deficiency on C57BL/6J mice causes red/pink blonde hair compared to a dark/black heavy pigmented complexion in wild-type state. The over-expression of MC1R causes a heavily melanized epidermis that might lead to deficiency in vitamin D production that requires UV-exposure for its biosynthesis (Slominski, Kim et al. 2013). Polymorphisms of MC1R were selected in world populations as people moved from the equator to geographical areas that UV is less rich to prevent 1α,25-Dihydroxyvitamin D3 (vitamin D) deficiency and rickets. For example, in Northern America, loss of function of MC1R is common. The most common MC1R polymorphism are known as red hair color (RHC) mutations Asp294His (D294H), Arg151Cys (R151C) and Arg160Trp (R160W) (Davies, Randerson-Moor et al. 2012, Pellegrini, Fargnoli et al. 2012). These mutations increase the incidence risk for sunburn and an increase prevalence of melanoma by four fold.

THE MC1R PATHWAY

The endogenous agonist ligand of MC1R is α-melanocyte stimulating hormone, α-MSH. The binding of α-MSH to MC1R leads to the activation adenylate cyclase (AC) (Rouzaud, Kadekaro et al. 2005). Then, AC converts a molecule of ATP to cAMP working as a second messenger protein with many roles in cellular signaling. This leads to the activation of the cAMP respond element-binding protein (CREB) to translocate to the nucleus inducing microphthalmia inducer transcription factor, MITF. This is a critical regulator for melanocyte differentiation and melanin production (Fig. 1-7).

THE TRANSCRIPTION FACTOR MITF

MITF is a transcriptional factor that recognizes E-box and M-box sequences in the promoter regions of target genes (Hoek, Schlegel et al. 2008). MITF regulates the expression of Tyrosinase, and Tyrosinase related proteins that are the main regulators in the synthesis of eumelanin. The accumulation of cAMP induces pro-differentiation and
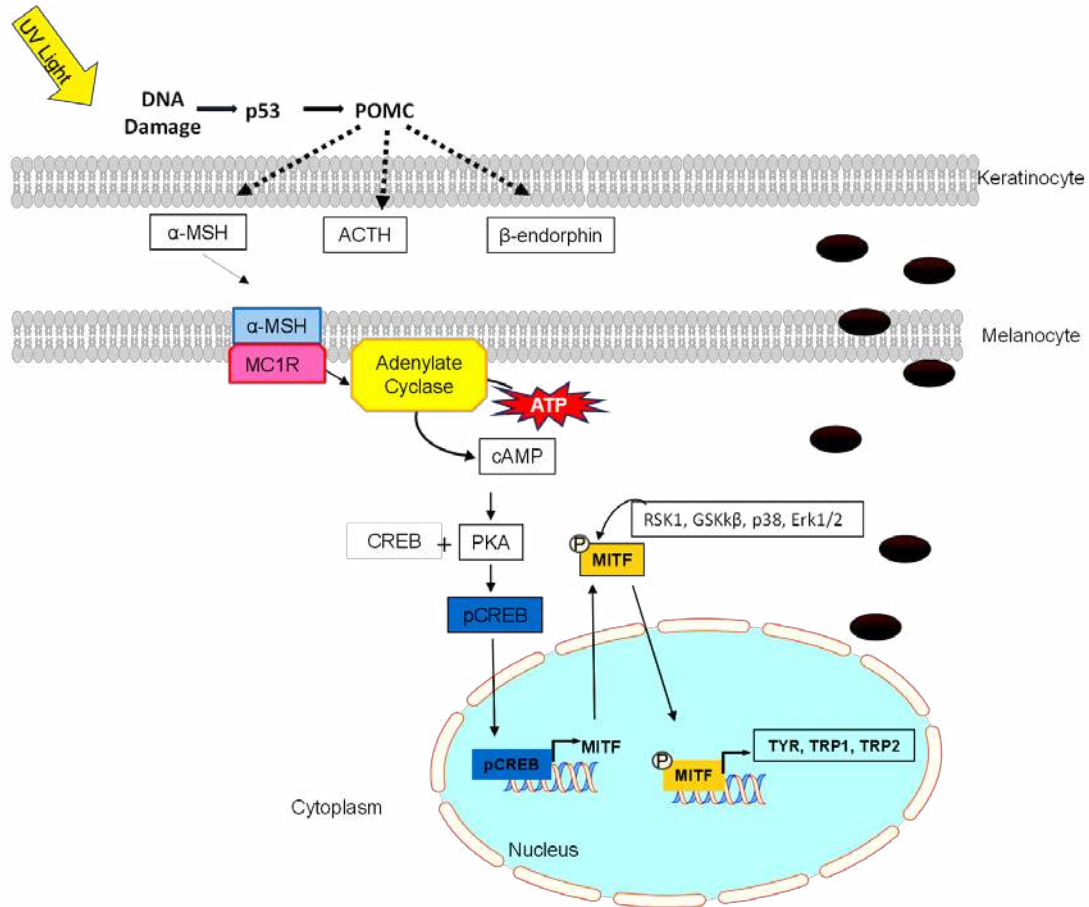
Modified with permission from D'Orazio, J. A., T. Nobuhisa, R. Cui, M. Arya, M. Spry, K. Wakamatsu, V. Igras, T. Kunisada, S. R. Granter, E. K. Nishimura, S. Ito and D. E. Fisher (2006). <u>Nature</u> **443**(7109): 340-344 and Cui, R., H. R. Widlund, E. Feige, J. Y. Lin, D. L. Wilensky, V. E. Igras, J. D'Orazio, C. Y. Fung, C. F. Schanbacher, S. R. Granter and D. E. Fisher (2007). <u>Cell</u> **128**(5): 853-864.

Figure 1-7. The adaptive tanning response.

Upon UV irradiation, the DNA damage response lead to the activation of the expression of POMC in keratinocytes. POMC is cleaved, producing three products, among them α-melanocyte stimulated hormone (α-MSH). Activated by its agonist α-MSH, MC1R promotes cAMP second messenger generation which induces melanocyte differentiation and survival pathways involving PKA, CREB and MITF In this way, cAMP induces both melanin production and antioxidants that reduce cellular ROS. cAMP, cyclic adenosine monophosphate. PKA, protein kinase A. pCREB, phosphorylated cAMP response binding element. ROS, reactive oxygen species. MITF, microphthalmia (Mitf) transcription factor.

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survival signaling in melanocytes in addition to high levels of MITF. Studies performed in B16 mouse melanoma cells confirmed a link between MITF expression and melanin production and stimulation of NADPH oxidase 4 (Nox4). Liu and colleagues showed that APE-1 induces MITF leading to cell survival (Liu, Fu et al. 2009). Low levels of cAMP led to lower expression of MITF and the synthesis of the oxidation-prone pheomelanin. Waardenburg syndrome (WS) is a genetic disorder arising from the neural crest and among its defects, there is deafness and pigmentation abnormalities. Deficiency in MITF expression has been identified in WS (Grill, Bergsteinsdottir et al. 2013). Kim and colleagues found that ROS induced ERK signaling increases MITF degradation (Kim, Park et al. 2014).

THE LIGANDS OF MC1R

α-MSH is produced in keratinocytes and is a metabolite of the pro-opiomelanocortin pro-peptide (POMC) (Hadley and Haskell-Luevano 1999). DNA damage pathways, including p53, induce POMC expression after UV injury. α-MSH is a small peptide with only 13 amino acids ($HOOC-SYSMEHFRWGKPV-NH_2$) (Sahm, Olivier et al. 1994). However, among soluble ligands of MC1R, α-MSH does not have the highest binding affinity. There are other products that are cleaved from POMC. These ligands are β-MSH, γ-MSH, β-endorphin and adrenocorticotropic hormone (ACTH) and they not only regulate weaker levels of cAMP with MC1R but also bind to other melanocortins expressed in the skin. There are two peptides identified that limit or inactivate MC1R signaling in the skin: agouti signaling protein (ASIP) and β-defensin 3 (BD3). Both β-defensin and agouti inhibit α-MSH binding MC1R by blocking its active site (Swope, Jameson et al. 2012). ASIP has higher affinity for MC1R than α-MSH and can directly bind and down-regulate the production of cAMP (Voisey and van Daal 2002, Ito and Wakamatsu 2011). BD3 was identified as an antimicrobial peptide and it has higher affinity to MC1R than ASIP (Abdel-Malek and Supp

2008, Swope, Jameson et al. 2012, Benato, Dalla Valle et al. 2013). It is important to remember that although MC1R has a protective effect in the skin, there is tight regulation of its activation and downstream targets. Although MC1R RHC mutations are known to impair MC1R signaling, there are different ligands that have a role as antagonists leading to MC1R inactivation. There is little known about the role of these antagonists but they might be present as a regulatory aspect to limit the production of melanin only when required. Nonetheless, MC1R is a key receptor for the regulation of adaptive pigmentation in the epidermis and its activation and regulation in the context of whole skin is likely to be complex.

MC1R DEFICIENCY

MC1R activates the adaptive pigmentation pathway. The phenotype of individuals lacking MC1R show a fair-skin complexion leaving the skin to the exposure of hazardous UV doses that could be blocked by the accumulation of more of eumelanin. This leads to DNA damage that contributes to melanocyte mutagenesis. Several studies show a direct impact of MC1R deficiency in the level of UV-damage products in melanocytes (Jarrett, Horrell et al. 2014). MC1R defective melanocytes are not able to repair UV damage efficiently leading to higher risk of mutagenesis skin-prone population. There are limited studies about the role of MC1R in the regulation of UV-induced oxidative injury. Mitra and colleagues reported that melanocytes with mutated MC1R lead to melanoma formation without UV exposure by the oxidative damage caused by the expression of pheomelanin (Mitra, Luo et al. 2012). The MC1R protein is also a major regulator of melanoma risk in humans (Valverde, Healy et al. 1996, Rees 2000, Box, Duffy et al. 2001, Kennedy, ter Huurne et al. 2001, Sturm 2002, Galore-Haskel, Azizi et al. 2009, Hoiom, Tuominen et al. 2009, Ibarrola-Villava, Fernandez et al. 2010, Scherer, Nalls et al. 2010, Cust, Goumas et al. 2012, Ghiorzo, Bonelli et al. 2012), therefore I have been interested in the mechanisms

by which MC1R signaling protects melanocytes against UV-induced oxidative stress and antioxidant defenses.

K14-SCF TRANSGENIC MOUSE MODEL

In humans, melanocytes are localized in the stratum basale above the basal lamina of the epidermis (Fig 1-1). The survival and migration of melanocytes is regulated by a receptor tyrosine kinase cKIT or KIT receptor (Murakami, Matsumoto et al. 1995, Herraiz, Journe et al. 2011, Azevedo, Horvath et al. 2013). This receptor controls melanocyte development, migration and survival and it is activated by a stem cell factor (*SCF*) also known as KIT ligand (Guo, Jie et al. 2014). The cKIT signaling pathway promotes phosphorylation of MITF leading to its short-lived activation and further degradation (Torres-Cabala, Wang et al. 2009). This regulates MITF expression after α-MSH signaling activates MC1R.

The use of mice as an animal model to investigate pigment-dependent physiology in the skin is limited because of innate differences between human skin and mouse skin. Whereas human skin maintains melanocytes in the epidermis throughout life (imparting melanin pigments to the epidermis), mouse skin is only transiently populated by melanocytes in the epidermis in the neonatal period. For adult mice, their melanocytes are located in the hair follicle at the dermis. This feature results in a fur coat color rather than skin pigment. In addition, during postnatal development, melanocytes can be found in the ears, tail and paws. However, these melanocytes cannot be retained due deficiency in stem cell factor (*SCF*) expression leading to inactivation of cKIT receptor.

One approach to be able to utilize mice to study pigment effects (such as cAMP rescue of melanization) is to transgenically express melanocyte growth factors (such as *SCF*) in the epidermis constitutively throughout life. We had reported the use of a transgenic C57BL/6 mouse model that resembles human skin. In this model, the keratin 14 (*K14*) *SCF*

transgene, which is constitutively expressed in keratinocytes, retains melanocytes in the epidermis. This model features localization of melanocytes in the epidermis and accumulation of black/dark pigmented melanin in the skin of wild-type $Mc1r^{E/E}$ $Tyr^{+/+}$ mice. On the other hand, there is production of a red/light pigmented melanin in $Mc1r^{e/e}$ $Tyr^{+/+}$ mice due to deficiency in MC1R signaling. There is an albino mouse $Mc1r^{E/E}$ $Tyr^{c2j/c2j}$ mice that signals MC1R, but it is deficient in tyrosinase expression, and it is unable to produce melanin. Finally, the albino *extension* mouse is $Mc1r^{e/e}$ $Tyr^{c2j/c2j}$ unable to produce melanin and it is deficient for MC1R signaling. This mouse model reflects three possible phenotypes in human skin complexion.

FORSKOLIN

There are numerous options for the induction of sunless tanning response in the skin. Among them, a natural product called forskolin has generated interest from researchers and general public. Forskolin, identified in 1974 by a Finnish botanist named Forskal, is a bioactive constituent of the root portion of the *Coleus forskohlii* (*Plectrantus barbatus*) plant (Lukhoba, Simmonds et al. 2006) and it is cultivated in South East Asia that included countries like India, Nepal, Sri Lanka, and Thailand. Scientists characterized it because the plant from which it was derived had long been used in traditional Ayurvedic medicine for a range of disorders and diseases. Forskolin, also known as Coleonol, has a unique heterocyclic structure that classifies the molecule as part of the labdane diterpene family (Fig 1-8).

The crude root forskolin extract has many components including alkaloids, phenols, tannins and histamines (Alasbahi and Melzig 2012). The forskolin extract has been reported to induce hypotension (De Vries, Amdahl et al. 1988, Lindgren, Crossley et al. 2011), blood thinness (Hayashi and Sudo 2009), mast cell degranulation (Cheli, Giuliano et al. 2012) and stimulation of adenylyl cyclase (Pinto, Papa et al. 2008). Forskolin has
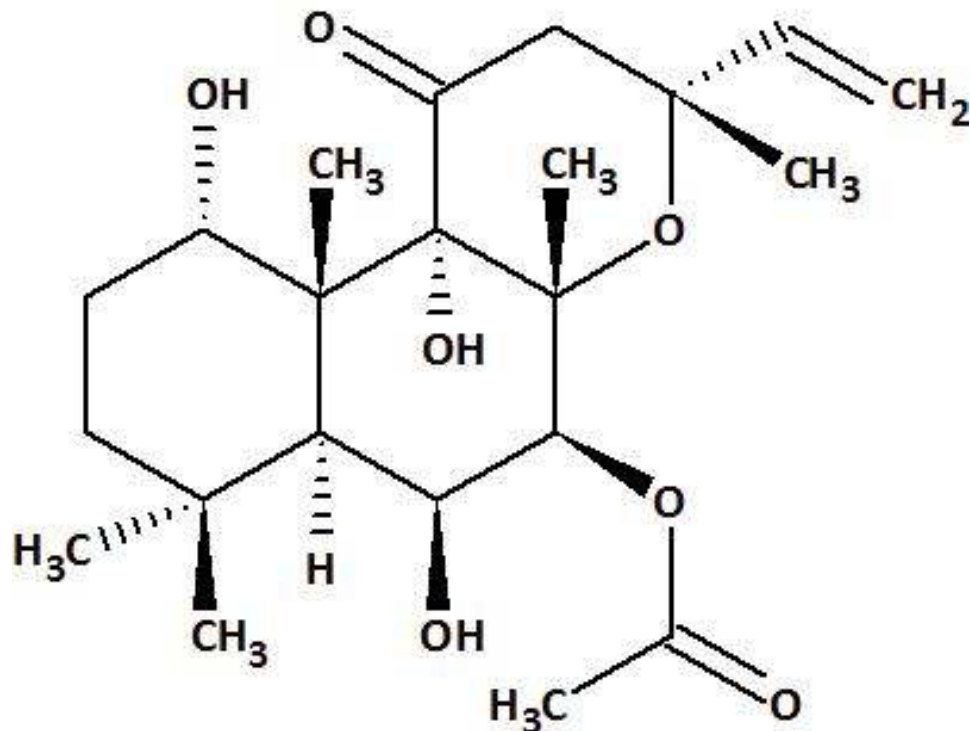
Figure 1-8. The structure of forskolin.

The compound, which molecular formula is $C_{22}H_{34}O_7$ and is known as forskolin, can bind to 8 of the 9 isoforms of adenylate cyclase (AC). AC is activated by G-protein to catalyze the conversion ATP to cAMP. Pharmacologic activation of AC using forskolin decreases receptor agonists' desensitization that down-regulate AC signaling cascade..

been study for its many beneficial physiological effects on skin conditions such as psoriasis and eczema. Recent studies attribute positive effects to treat weight loss (Geller, During et al. 1993, Doseyici, Mehmetoglu et al. 2014) and to alleviate symptoms associated with painful menstrual periods (da Conceicao, de Oliveira et al. 2012). Together with antibiotics, forskolin has been used for the treatment of urinary tract infection (Doseyici, Mehmetoglu et al. 2014). Forskolin has been studied as a vasodilator for cardiovascular diseases to prevent hypertension (Enomoto, Yoshihisa et al. 2011). However, oral administration of forskolin also induces side effects such as headaches and accelerated heart rate (Galeotti, Ghelardini et al. 2001). These effects have been linked to an increase in bleeding episodes (Onoue and Katusic 1998).

At the molecular level, forskolin is able to bind to adenylate cyclase (AC), an enzyme responsible for the production of the second messenger cAMP from ATP. The AC isoforms are restricted to specific tissue (brain, liver, lung, uterus, testis and muscle) and excitable cell types (endothelial cells) (Sunahara and Taussig 2002). There are nine isoforms of AC and the binding of G-protein subunits regulates its activation. Forskolin is able to mimic the G-protein and it binds at the catalytic site of AC to allow ATP access and transformation to cAMP (Cumbay and Watts 2004). Forskolin can bind to all AC isoforms except AC9 (Cumbay and Watts 2004). Once activated AC changes its conformation to allow the binding of ATP. ATP is converted to cAMP which is a second messenger inducer for a numerous of intracellular signaling pathways. There are pharmacologic drugs available that can directly increase cAMP levels such as forskolin and rolipram, a phosphodiesterase (PDE) inhibitor. PDE is known to mediate the degradation of cAMP. These drugs can activate the pathway independent of MC1R status. However, a pharmacologic formulation of forskolin is able to penetrate the skin and is efficiently absorbed transdermally.

FORSKOLIN-MEDIATED MELANIN RESCUE

As discussed previously, MC1R triggers the activation of adenylate cyclase for the production of cAMP, activation of MITF, and synthesis of protective eumelanin. However, deficiency in MC1R signaling induces low levels of cAMP and production of pheomelanin. However, MC1R RHC mutation and inhibitors do not affect the mechanism mediated by forskolin since forskolin is able to bypass the receptor dependent signaling (Abdel-Malek and Supp 2008). We, together with other laboratories, have utilized forskolin as an adenylate cyclase activator. Using our mouse model, topical administration of forskolin induced eumelanin accumulation in the skin of *K14-SCF* Mc1r$^{e/e}$ Tyr$^{+/+}$ *extension* mice compared with non-transgenic treated and untreated mice. After chronic stimulation of cAMP daily for 21 days leading to accumulation of melanin, these mice showed no changes in body weight and liver size (Spry, Vanover et al. 2009). However, there was an increase in epidermal thickening (Scott, Christian et al. 2012). Forskolin induced expression of the keratinocyte growth factor, leading to keratinocyte accumulation in the epidermis.

Therefore, among its other mechanisms, forskolin-induced skin darkening is a target of cAMP production (D'Orazio, Nobuhisa et al. 2006). Topically-applied forskolin seems to be systemically absorbed, leading to systemic cAMP stimulation in the mice as evidenced by darkening of skin in other sites. Previously, we reported that the most darkening of the skin was obtained after 2 weeks of daily treatment with forskolin and persist as long at the topical application was continued. In fair-skin individuals, unless there is constant application of forskolin, the darkening of the skin fades away and the skin returns to its normal shade. This means that the *K14-SCF* by itself does not rescue melanin production of the skin. The mechanism of skin darkening requires stimulation of the adaptive pigmentation pathway for the production of melanin and further deposition in

keratinocytes. If there is pharmacologic stimulation of melanin production, we can study the relation between the stimulation of MC1R and the expression of UV-mediated DNA damage products after darkening of the skin. In addition, this method is useful to study DNA repair mechanisms that can be addressed as targets for the therapeutic application and prevention of UV-mediated skin mutagenesis.

MELANIN-INDEPENDENT EFFECTS

There are other effects mediated by forskolin that are melanin-independent. The solute carrier family 24 member 5 (SLC24A5) is a regulator of the ratio of dark and light pigmented melanin in the skin. Cheli and colleagues were able to show the up-regulation of this transporter after an increase of cAMP in forskolin treated B16 mouse melanoma cells (Cheli, Luciani et al. 2009). This regulation has an important role in melanosomes alkalization and regulation melanin synthesis. Spindler et al. studied the role of cAMP in the loss of keratinocytes cohesion and further blister formation in transformed keratinocytes and neonatal mice (Spindler, Endlich et al. 2011). Forskolin together with rolipram, prevented the loss of desmoglein, a calcium-dependent adhesion molecule, preventing the loss of cell adhesion. Kokot and colleagues study the effect of the MC1R/cAMP pathway in the regulation of antioxidant enzymes expression (Kokot, Metze et al. 2009). The use of α-MSH induces Nrf2 and Nrf2-induced gene expression in human keratinocytes. Nrf2 is a transcription factor for key antioxidant enzymes such as glutathione and glutathione regulators. Forskolin shows the same effect as α-MSH inducing expression of Nrf2 by increasing cAMP production and CREB binding to the promoter region of Nrf2 (Kokot, Metze et al. 2009). Furthermore, forskolin increases the removal of cyclobutane pyrimidine products and 6-4PP caused by UVB exposure (Jarrett, Horrell et al. 2014). A proposed mechanism of action includes the modulation of DNA damage repair factors such as xerodermal pigmentosum complement group A (from the

NER pathway) as a downstream target of cAMP signaling. In addition, forskolin treatment of human keratinocytes decreased UVB-induced apoptosis (Passeron, Namiki et al. 2009).

RATIONALE

UV exposure is one of the most important environmental health hazards, clearly causative for age-related skin changes such as wrinkling, pigmentary changes, thinning and carcinogenesis. Because of complex societal factors, UV exposure may actually be increasing through increased occupational and recreational activities including indoor tanning. There is emerging evidence implicating MC1R and cAMP signaling in regulating antioxidant proteins. Using keratinocytes transfected with MC1R, Henri et al. reported lower cellular levels of ROS after pharmacologic activation MC1R/cAMP pathway and higher levels of ROS when PKA was pharmacologically inhibited (Henri, Beaumel et al. 2012). In other work using human melanocytes, Song and colleagues found that α-MSH-induced MC1R signaling increased levels of catalase after UV exposure (Song, Mosby et al. 2009). Finally, Kaderaro and coworkers reported that cAMP stimulation reduced levels of hydrogen peroxide, an important ROS, in human melanocytes after UV exposure (Kadekaro, Chen et al. 2012).

Loss of function MC1R polymorphisms impedes accumulation of protective melanin in the skin. MC1R deficiency is linked to skin cancer incidence. As we learn more about innate signaling mechanisms that regulate natural antioxidant defense pathways in the skin such as the MC1R hormonal axis, new approaches are being designed to exploit these signaling pathways to delay or even prevent free-radical induced symptoms of aging. Use of natural extracts such as forskolin enhance protection against UV-induce molecular damage to the skin. cAMP-induced melanin deposition and antioxidant induction may prove to be an important therapeutic opportunity to reduce UV-mediated pathologies.

PROJECT OBJECTIVES

In this study, I explore the role of the MC1R pathway in protecting against UV-induced sunburn and providing skin protection by enhancing expression of antioxidant enzymes in the skin. We had previously demonstrated that the topical application of a cAMP activator highly activates the MC1R pathway in Mc1r$^{e/e}$ mice.

This study addresses the following issues in two chapters:

Chapter 2: MC1R signaling pathway promotes epidermal pigmentation and protect against sunburn.

Chapter 3: MC1R signaling pathway increases MnSOD levels is the mitochondria and does not affect mitochondrial oxidative phosphorylation.

In chapter two, I hypothesize that a short-term topical activation of MC1R/cAMP pathway decreases UV sensitivity in Mc1r$^{e/e}$ Tyr$^{+/+}$ mice. Our laboratory previously reported the development of a *K14-SCF* mouse model with a robust deposition of black melanin pigment due to the presence of epidermal melanocytes. Mc1r-defective fair-skinned mice resulted in an accumulation of eumelanin and a UV-protected phenotype against skin sunburn. In addition to this phenotypic effect, I studied the role of the MC1R pathway to protect the skin at the molecular level.

In chapter three, I hypothesize that MC1R signaling protects against oxidative stress by the enhancement of antioxidant cellular defenses. These results provided an insight about a possible mechanism of MC1R signaling pathway to control biosynthesis and regulation of antioxidant enzymes by enhancing their gene expression. Moreover, I explore the protein levels of the MnSOD in human melanocytes. The results suggest that increases MnSOD may not involve transcriptional up-regulation but rather MnSOD transport and/or stability. Furthermore, I studied if there was any protective effect in mitochondria metabolism after activation of the MC1R/cAMP signaling pathway.

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CHAPTER 2

MC1R signaling pathway promotes epidermal pigmentation and protect against sunburn.

This chapter is based on work published as Amaro-Ortiz, A., Vanover, J.C., Scott, T.L., D'Orazio, J.A. Pharmacologic Induction of Epidermal Melanin and Protection against Sunburn in a Humanized Mouse Model. J. Vis. Exp. (79), e50670, doi: 10.3791/50670 (2013).

INTRODUCTION

The most deadly form of skin cancer is melanoma. In the United States, there is a dramatic increase in the incidence of melanoma among light-skinned individuals. Epidemiologic evidence showed a 20 % increase in the frequency of melanoma in fair-skinned individuals compared to dark-skinned individuals. UV radiation is a major environmental factor that contributes to the increased risk to develop skin cancer. Long exposure to solar radiation and the constant use of tanning beds increase the intensity of UV exposure to the skin. UV radiation induces direct and indirect molecular damage that can lead to genetic mutations. These mutations lead to proliferative skin cells that spread to other parts of the body and produce the generation of tumors. In addition, early life episodes of skin sunburn have been linked to an increase in melanoma risk. Still, skin pigmentation has a mayor role to determine the risk of developing melanoma.

The ratio of dark eumelanin to reddish pheomelanin varies among individuals. I focused on fair-skinned individuals with low levels of eumelanin because more UV photons can penetrate into the skin. There is a direct correlation between melanoma risk and the signaling ability to "tan" following UV exposure. The activation of the melanocortin 1 receptor (MC1R) pathway increases the levels of protective eumelanin in the skin. The MC1R is a G-protein couple receptor located in the plasma membrane of melanocytes

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and mediates activation of adenylate cyclase to increase production of cAMP. The secondary messenger cAMP allows activation of main regulators of melanin biosynthesis as previously discussed. UV mediates MC1R activation allowing melanocytes differentiation and melanin production. Melanin is a complex polymer produced in melanocytes is deposited in keratinocytes to protect the skin against exposure to higher and hazardous UV doses. The endogenous ligand of MC1R is the α-melanocytes stimulating hormone, α-MSH. The ligand is a tripeptide that can activate the MC1R and induce the tanning response thus reducing UV-induced DNA damage in human melanocytes. However, the down-regulation of the MC1R pathway increases the levels of non-protective pheomelanin in the skin. I was interested in the mechanism by which MC1R mediates melanocyte protection against UV-exposure. I used *extension* mice ($Mc1r^{e/e}$ $Tyr^{+/+}$) that model the fair-skinned humans with decreased MC1R signaling. This transgenic model constitutively expressed *K14* stem cell factor (*SCF*) allowing epidermal melanocytes to be retained at the basal layer of the epidermis. However, non-transgenic mice maintain its melanocytes on the dermis in hair follicles.

Our laboratory developed an animal model to address the mechanism of the MC1R pathway. For this study, the mice used were wild type mice ($Mc1r^{E/E}$ $Tyr^{+/+}$) darker-skinned mice; *extension* ($Mc1r^{e/e}$ $Tyr^{+/+}$) fair-skinned mice due to decreased MC1R signaling. Our previous research confirmed a melanizing effect of chronic topical treatments of forskolin correlating with increased UV-resistance in *extension* ($Mc1r^{e/e}$ $Tyr^{+/+}$) *K14-SCF* animals. In this chapter, I demonstrate that short-term application of forskolin also rescues MC1R signaling pathway. Moreover, I measured the deposition of melanin pigment and tested protection against UV-induced sunburn by measuring the minimal erythematous dose, MED.

SPECIFIC AIM:

To study the rescue of MC1R signaling pathway using forskolin

a) To determine the role of forskolin after a short-term topical application.

b) To determine the deposition of melanin after MC1R stimulation.

c) To examine how MC1R activation protects the skin against sunburn.

METHODS AND MATERIALS

Protocols for murine experiments followed the guidelines for ethical conduct in the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Protocol # 00768M2004).

MOUSE COLONY

4 to 12 week old C57BL/6 *extension* non-transgenic and *K14-SCF* transgenic *extension* males and females mice were used. The characteristic phenotype was blonde fur, pink skin, dark footpads and nose tip. The mice showed pheomelanotic fur, pheomelanotic epidermal skin (due to presence of interfollicular epidermal melanocytes).

REAGENTS AND MATERIALS

The following reagents were purchased commercially: Coleus Forskohlii 20% extract (Buckton Scott USA Inc., Princeton NJ); Depilatory cream Nair (Church & Dwight, Princeton , NJ); Xylazine (Anased Injection, Shenandoah, IA); Ketamine (Putney, St. Joseph, MO.); Isothesia Isoflurane (Butler Schein, Dublin, OH); Propyl glycol (Amresco, Solon, OH) and Ethyl Ethanol (Fisher, Waltham, MA.). The following materials were purchased commercially: Chromameter CR-400 and data processor (Konica Minolta, Ramsey, NJ); Electric Shears (Oster, Atlanta, GA); NIST Radiometer/Photometer Model IL1400A, International Light, Newburyport, MA), and Germicidal Hg Lamb UV-B (Westinghouse, Pittsburgh, PA).

The preparation of forskolin was made by solubilizing P. barbatus root extract 20% (ATZ Natural, NJ) The root extract is prepare at 40% weight/volume in a standard dermatologic base of 70% ethanol, 30% propylene glycol. After stirring for an hour at room temperature, the slurry was centrifuged (1,500 x g, room temperature, for 15 minutes). The solution was filtered through a 0.22µm cellulose acetate membrane to remove any residual insoluble material from the extract. The extract maintained biologic activity for months at room temperature.

PREPARATION OF C57BL/6 *K14-SCF* MICE FOR TOPICAL TREATMENTS

The mice were briefly anesthetized using intraperitoneal injection of a standard mixture of ketamine (Putney, St. Joseph, MO.) and xylazine (AnaSed, Shenandoah, IO.) (Typically 0.04ml per 10g body weight of a mixture of 10mg/ml ketamine and 1.0mg/ml xylazine). The dorsal fur was removed using electric shears equipped with a 0.25mm surgical preparatory head followed by chemical depilation by Nair (Church & Dwight, Princeton, NJ.). After 24 hours, the mice were treated in the dorsal skin with 400uL of 40% crude forskolin extract; vehicle control animals received 70% ethanol, 30% propylene glycol alone twice daily for 5 days (10 applications). Then, 48 hours after the last topical treatment, the mice were anesthetized with ketamine and xylazine so that UV sensitivity by calculation of "Minimal Erythematous Dose" (MED) could be determined. A UV-occlusive tape was placed on the back of the mice. The mice were placed in a UV source consisting of two Westinghouse F15T8UV-B lamps with a peak output of 313nm and a range of 280 to 370nm based on the UV transmission rate as measured by the UV Photometer. The UV exposure time was calculated for each desired dose based on the UV output of the source. The mice were monitored for 24 to 48 hours to look for discreet

areas of erythema (redness) or edema (swelling) corresponding to the anatomic sites exposed to the specific dose of UV irradiation. The skin findings were documented photographically. The skin color was measured by reflective colorimetry while the mouse was briefly anesthetized by inhaled isoflurane (Butlet Schein, Dublin, OH). A Minolta colorimeter was calibrated by placing the portable head on the standardized white surface provided with the colorimeter.

STATISTICAL ANALYSIS

The data was analyzed between cohorts of replicate samples (n = 3) by one-way ANOVA using the Bonferroni post-test (Graph Pad PRISM software). p values <0.05 were considered statistically significant.

RESULTS

FORSKOLIN-INDUCED MELANIZATION OF FAIR-SKINNED MICE.

I tested the rescue of the MC1R signaling pathway in the C57BL/6 *extension* non-transgenic and *K14-SCF* transgenic *extension* pheomelanotic ($Mc1r^{e/e}$, $Tyr^{+/+}$) mice (Fig. 2-1A). The ears of the mice show the retention of melanocytes in the epidermis. The mice were treated twice daily for 5 days with either vehicle (70% ethanol, 30% propylene glycol) or 40% crude Coleus Forskohlii root extract (Fig. 2-2 B). The amount of melanization of the skin was dose dependent. The concentration of forskolin was 80uM per dose per application. The effects of topical application on the dorsal skin were inspected visually and using a reflective colorimeter (Fig. 2-2B). I reported an accumulation of robust epidermal darkening after the last application of the root extract in the C57BL/6 *K14-SCF* transgenic *extension* mice but not in the C57BL/6 *extension* non-transgenic mice. These results indicate the importance of the location of epidermal melanocytes in order to allow pharmacologic stimulation of melanin to be deposited in the keratinocytes located in the epidermis. Although the root extract contains plant photo-chemicals that contribute to its

color, there is no skin darkening in non-transgenic animals. Therefore, the dyeing effect from the drug has no contribution to the skin darkening (Fig. 2-2A). The topical treatment of forskolin showed an effect as early as two days and continued to a maximal darkening after several applications (Fig 2-2B).

TOPICAL FORSKOLIN DECREASES UV SENSITIVITY.

Next, I determined the effect of the topical application of forskolin on UV sensitivity by testing MED in C57BL/6 *K14-SCF* transgenic *extension* mice. I compared this group with non-transgenic mice as a control for the non-forskolin component of the root extract. The reflective colorimetric white-black scale L* value was 31.9 ± 1.8 kJ/m^2 compared to 3.8 ± 1.8 kJ/m^2. Actually, a dose of 30 0 kJ/m^2 did not generate erythema in the dorsal skin. This was expected since the mice show no obvious side effects after the scheduled 10 applications; thus, I concluded that twice-daily administration of forskolin induces a non-toxic and safe melanization. The MED was measured 2 days after the last topical application of forskolin, thus non-pigmentary effect of cAMP have no role in MED results (Fig 2-3A,B). Thus, whereas mean MED for *K14-SCF extension* mice treated for twice for 5 days with vehicle was 5.0 ± 0.0 kJ/m^2, average MED for cohorts treated with topical forskolin was $> 30.0 \pm 0.0$ kJ/m^2. In fact, a dose of 30.0 kJ/m2 was insufficient to generate erythema in this experiment. There was no significant difference when I compared non-transgenic and *K14-SCF* transgenic *extension* treated with vehicle (70% ethanol, 30% propylene glycol). I reported an L* value of 3.3 ± 1.4 kJ/m^2 compared to 5.0 ± 0.0 kJ/m^2, respectively. Overall, forskolin induces epidermal melanization and provides a decrease in UV sensitivity in fair-skinned individuals.
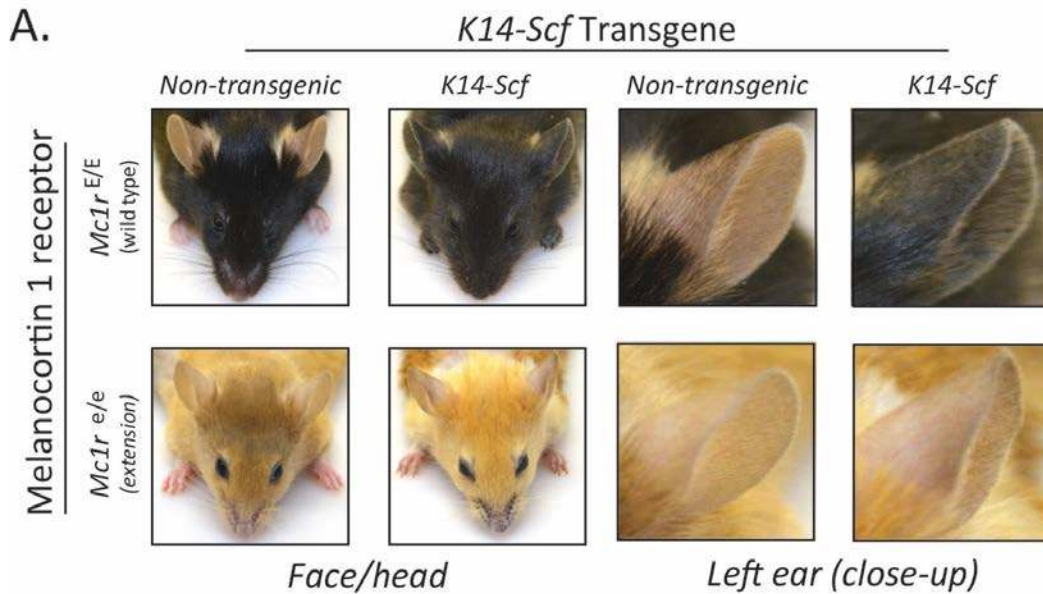
Figure 2-1. Topical treatment of forskolin promotes skin darkening in fair-skinned *extension*.

 (Mc1r^e/e) mice photographs of C57BL/6 animals used in this study. Animals are genetically matched except at the melanocortin 1 receptor (Mc1r). Note that pigmentation is eumelanotic (black) when Mc1r is functional but pheomelanotic (blondish) when Mc1r is defective, as is the case with the *extension* (Mc1r^e/e) mutant. Epidermal pigmentation depends on retention of interfollicular epidermal melanocytes in the skin by the *K14-SCF* transgene, and the skin darkening can easily be seen in the ears.
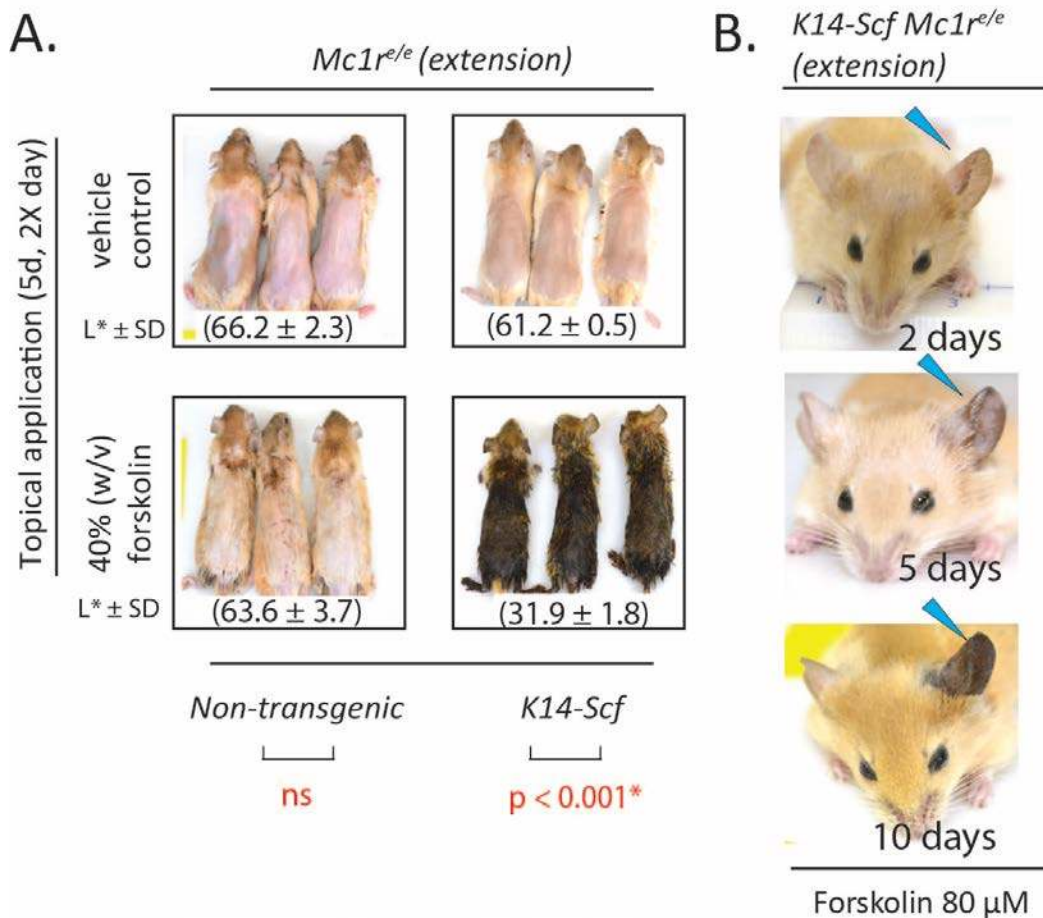
Figure 2-2. Twice daily application of Forskolin induces MC1R signaling.

(A) Photographs of *extension* (Mc1r[e/e]) *K14-SCF* or non- transgenic animals treated with 400uL of vehicle control (70% ethanol 30% propyl glycol) or 40% w/v (80 µM) forskolin applied twice daily to the shaved dorsal skin for 5 days, total of 10 applications. Skin color measurements by reflective colorimetry were performed for each group. Reflective colorimetry results are reported as mean ($\pm$ SD) reflectometry units on the L* (white-black) color axis. Note that topical administration of forskolin caused robust skin darkening in *K14-SCF* transgenic animals but not in non-transgenic mice. (B) Time course experiment showing darkening of the forskolin-treated ear of *K14-SCF extension* mice for the indicated times (forskolin-treated ears are indicated by the blue triangles). Vehicle was applied to the right ear for comparison. The L* $\pm$ SD results are reported as reflective colorimeter white-black scale, * $p \leq 0.001$.

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A.

Twice daily application (5d)

0kJ/m²	⬤⬤	10kJ/m²
2.5kJ/m²	⬤⬤	20kJ/m²
5kJ/m²	⬤⬤	30kJ/m²

B.

$Mc1r^{e/e}$ (extension) $Tyr^{+/+}$

	vehicle control		Forskolin	
L* ± SD	(66.2 ± 2.3)	(61.2 ± 0.5)	(63.6 ± 3.7)	(31.9 ± 1.8)
MED	(3.3 ± 1.4)	(5.0 ± 0.0)	(3.8 ± 1.8)	(> 30)
	Non-transgenic	K14-Scf	Non-transgenic	K14-Scf
	ns	ns	ns	p < 0.001*
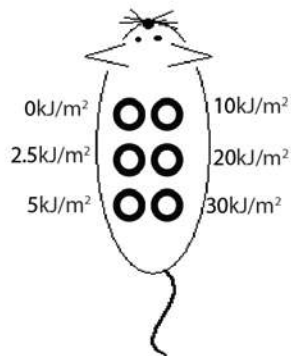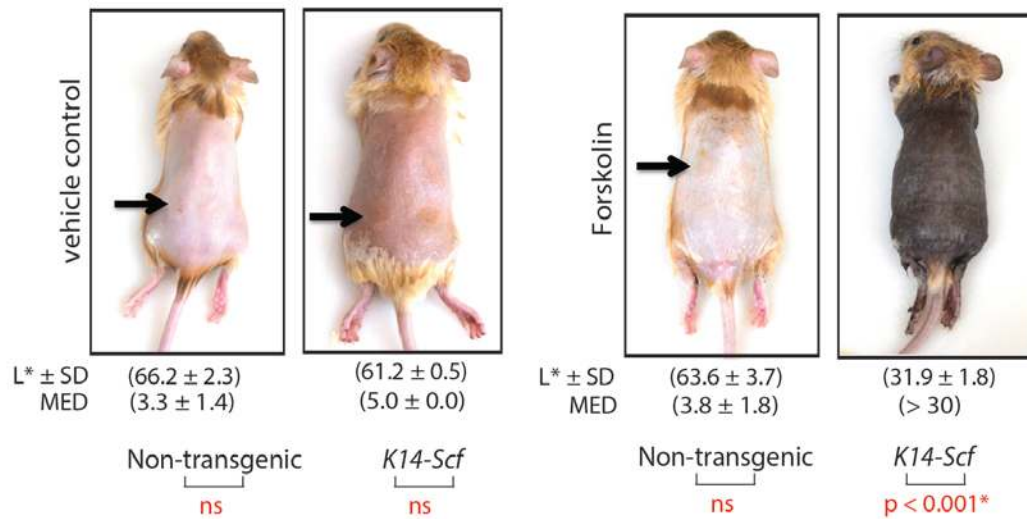
Figure 2-3. Forskolin-induced melanization protects against UV-mediated inflammation as determined by minimal erythematous dose (MED) testing.

(A, B) Position of UV occlusive tape and UVB doses of animals treated twice daily for 5 days. The last topical treatment was applied 48h prior to irradiation. Dorsal skin was exposed to various doses of UVB by using UV-occlusive tape with punched-out 1 cm² circular apertures, and varying exposure times to yield the appropriate dose. After irradiation, circles of exposed skin were labeled with a pen in some experiments. MED's, defined by erythema and/or edema of the entire circle of exposed skin to a particular dose, were determined 48 hours after exposure. The MED ± SD results are reported as kJ/m² UVB, * p ≤ 0.001.

DISCUSSION

It is vital to select a functional mouse model to study skin melanization. There are differences between human and mice skin such as thickness, epidermal melanocytes, and dermis structure. UV mediates expression of MC1R endogenous ligand α-MSH in keratinocytes. Nevertheless, in Mc1r$^{e/e}$ mice, there is no UV-mediated melanization due to MC1R deficiency, and these mice have higher levels of pheomelanin or red/blonde pigment than the dark pigment eumelanin. Similarly, there is no UV- or forskolin- induced pigmentation in *K14-SCF* non-transgenic mice. In that case, pharmacologic stimulation such as forskolin-mediated cAMP signaling of the adaptive pigmentation pathway provides UV protection for fair-skin mice.

UV sensitivity is a common characteristic in humans unable to tan after long exposure of solar radiation. The deficiency in the production of melanin allows higher dose of UVB to penetrate the skin and induce UV-damage. I hypothesized that the use of forskolin as a short-term treatment allows the accumulation of melanin in Mc1r-deficient mice. I used a mouse model of the fair-skinned individuals and I found that topically applied crude extract of forskolin induced skin darkening by increasing the synthesis of melanin production in the skin. This model mimics the complexion of human skin, retaining epidermal melanocytes compared to non-transgenic mice with dermal melanocytes unable to produce melanin. This was possible by the expression of the stem cell factor *K14* at the epidermal basal layer in transgene mice to mimic what occurs in human skin. The location of interfollicular melanocytes is key to obtain an accumulation in melanin. Our laboratory animal model include the *K14-SCF* transgene on C57BL/6 *extension* (Mc1r$^{e/e}$ Tyr$^{+/+}$). Mutated MC1R down-regulates the production of cAMP signaling leading to a pheomelanotic phenotype. However, *K14-SCF* Mc1r$^{e/e}$ Tyr$^{+/+}$ mice showed an abundant accumulation of protective eumelanin when treated with forskolin. Therefore, the use of a

pharmacologic stimulation of MC1R is able to rescue and balance the production of eumelanin in a fair-skinned background. The mechanism of activation of the MC1R has been well established. MC1R is able to induce cAMP production leading to pro-survival and pro-differentiation signals to melanocytes. However, direct activation of the adenylate cyclase is able to bypass mutations of MC1R signaling. I selected the crude root extract of forskolin as a natural option for a topical treatment against hazardous UV exposure because use of the purified forskolin was not cost-effective for topical treatments. Previous studies from our laboratory showed the use of 40% weight per volume solution of the root extract containing 80uM of forskolin. Here I showed that this dose induces darkening in the skin after the first two applications. I reported that twice-daily application is associated with a clear darkening of the skin providing significant UV protection. Compared to previous studies, our data reports that forskolin can be administrated more than once a day and be tolerated by the mice. There is no swelling of the skin after forskolin is applied. Therefore, the darkening of the skin is due to accumulation of eumelanin and not proliferation of the cells in the epidermis. When topical treatment were discontinued, the dorsal skin gradually returns to its pheomelanotic complexion.

Based on the data, pharmacologic activation of cAMP showed a significant increase in UV-protection as suggested by measuring the minimal erythematous dose (MED). The MED is the lowest dose of UV that induces erythema of the skin. The *K14-SCF* transgenic *extension* mice that were treated with forskolin showed > 6 fold increase in MED compared to non-transgenic *extension* mice. The use of topical stimulation of cAMP rescues melanin synthesis and induces MC1R-dependent signaling providing protection against UV-induced skin injury.

This study has many implications for the prevention of UV-induced skin damage. The skin is constantly exposed to environmental factors, of which solar radiation is a major

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carcinogen. Topical pharmacologic activation of MC1R/cAMP provides a possible therapeutic target against individuals unable to tan and who are prone to skin mutagenesis (D'Orazio, Nobuhisa et al. 2006). Forskolin induces darkening of the skin by activating the synthesis of endogenous melanin. The skin will fade back to its baseline skin complexion once the treatment is removed. Therefore, a persistent application is required to maintain darkening of the skin. Forskolin can effectively induce skin darkening and increase UV sensitivity by increasing MED in mice, establishing a proof-of-concept to test the ability of forskolin to rescue deficiencies in the MC1R/cAMP signaling pathway. The study of role of forskolin at the molecular level will provide a mechanism of action to determine how to use this potent drug as a topical treatment.

CHAPTER 3

MC1R signaling pathway increases MnSOD levels in the mitochondria and does not affect mitochondrial oxidative phosphorylation.

INTRODUCTION

This chapter is focused on the regulation of antioxidant defenses in melanocytes. In the skin, melanocytes are located in epidermis and the accumulation of UV-induce damage could lead to melanocyte transformation into melanoma. Antioxidant enzymes are the first line of defense against an increase in ROS. Interestingly, the cell produces endogenous levels of ROS that are able to regulate and to impair cellular signaling pathways. Moreover, mitochondrial production of endogenous levels of ROS in the cell is tightly regulated by antioxidant enzymes.

SODs are the only antioxidant enzymes that are able to interact with a radical and converted it to two different species, H_2O_2 and O_2. MnSOD is located in the mitochondria and knockdown of this enzyme leads to cell death. It is an essential protein regulated by NF-κB. It is not clear what happens once MnSOD engages to superoxide radicals but it is known that it can be inhibited by $OONO^-$ (Kim, Rodriguez et al. 2001). There are limited studies considering the role of MnSOD in the skin (Yan, Li et al. 1999, St Clair, Zhao et al. 2005, Luanpitpong, Chanvorachote et al. 2012, Holley, Xu et al. 2014), especially in melanocytes, the precursor of melanoma.

Preliminary data from our laboratory showed a correlation between the level of MITF and the presence of mtDNA damage in the cell. Different human melanoma cell lines were tested for the expression of MITF (Figure 3-1A). WM1366 and 451Lu were the cell lines with the highest protein levels for MITF and the lowest amount of mtDNA lesions. These lesions were measured by mtDNA amplification after UV exposure (Figure 3-1B). The small and circular mtDNA is more prone to mutation than nuclear DNA (Larsson and

Clayton 1995),(Nagley and Wei 1998). A faster amplification of different mtDNA fragments represents a higher frequency of DNA lesions. We found that the use of an oxidative agent such as H_2O_2 caused increases in mtDNA mutation in cell lines that expressed low levels of MITF (Fig. 3-1B). In addition, there was a decrease in the amount of mutation frequency present in human cell lines after UV exposure with rescue of MC1R/cAMP signaling (Fig. 3-1C, D). The cell lines were treated with chloramphenicol (CAP), an antibiotic whose mechanism of action allows study of mtDNA mutagenesis. CAP binds to mitochondrial ribosomes inhibiting mitochondrial protein synthesis and cellular proliferation. Treating cell lines with sub-lethal doses of UVB allows cells to incorporate mutations that will impair ribosome structure and interfere with CAP binding because of UV-induced DNA mutations to rDNA sequence. If mutations are generated after UV exposure, CAP cannot bind to ribosomes, and cells can grow as a result in its presence. After incubating cells with forskolin as a cAMP activator, the cells were irradiated. The cells that generated mutation on its DNA after UVB exposure were able to proliferate. Forskolin pretreatment decreased mutation frequency from 7×10^{-4} to 3×10^{-4} in A375 cell line (MC1R R151C) and from 6×10^{-4} to 2×10^{-4} in SBCL2 (wild-type MC1R) cells, suggesting that cAMP signaling protects against UV-mediated mitochondrial mutagenesis (Fig. 3-1C, D). We conclude that MC1R signaling has a protective effect against UV-induced mitochondrial mutagenesis.
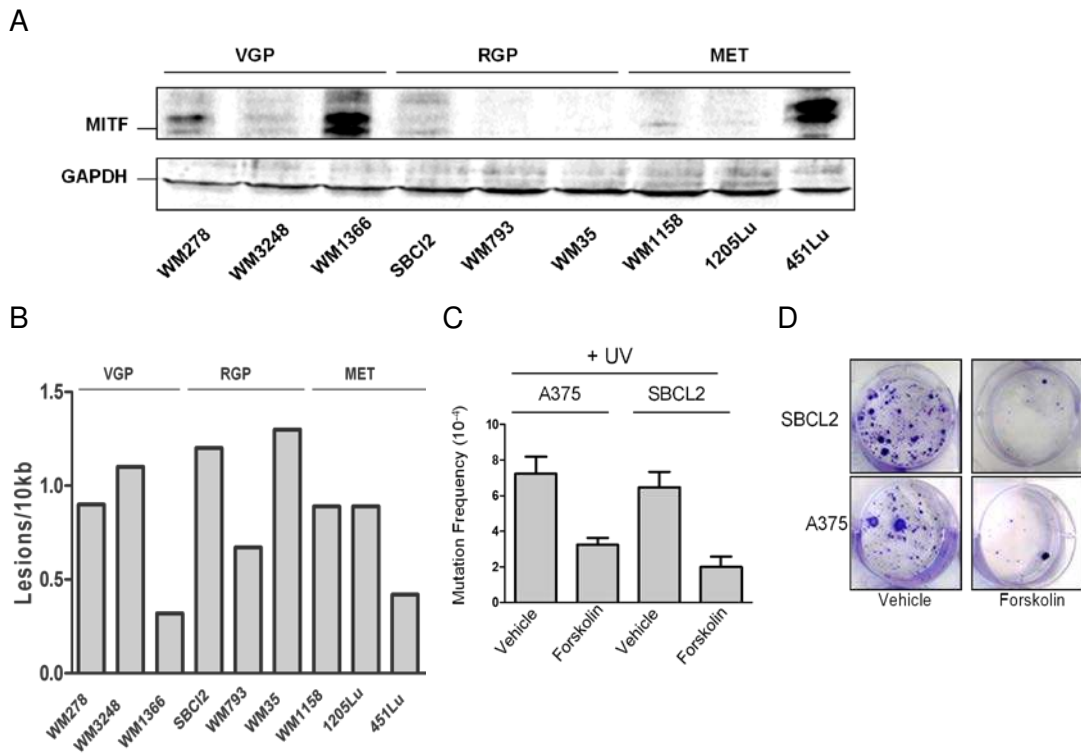
Figure 3-1. The level of mtDNA damage in melanoma cell lines.

(A) MITF expression in melanoma cells at various stages of progression. Cells were serum starved for 24h and whole cell lysate was probed for MITF levels. (B) H_2O_2-induced mtDNA damage in a panel of melanoma cells. Cells were serum starved for 24h and then exposed to oxidative stress (100 µM H_2O_2) for 30 minutes immediately following damage, DNA was isolated and XL-PCR performed. A 10kb primer set specific to the mitochondrial genome was used to determine levels of mtDNA damage. (C) Chloramphenicol (CAP) resistance assay for wild-type and mutated MC1R. Cells were treated twice to a sub-lethal dose of UV before incubation with CAP growth media for two weeks until cell colonies developed. (D) Representative pictures of colonies. VGP (Vertical growth phase), RGP (Radial growth phase) & MET (Metastatic phase)

Experiment performed in collaboration with Dr. Stuart Jarrett, PhD.

In this chapter, I studied the role of the rescue of MC1R pathway to enhance the expression of several genes that have a role in the oxidative stress regulation. Several studies confirmed that MITF plays a role in skin other than by inducing melanin production (Kim, Kim et al. 2005, Carreira, Goodall et al. 2006, Liu, Fu et al. 2009, Yi, Zhao et al. 2011, Berwick, MacArthur et al. 2014). Nonetheless, there is limited evidence of specific induction of the gene expression of antioxidant enzymes after activation of MITF in melanocytes.

In addition, I studied activation of MC1R signaling and its role in the translocation of the antioxidant enzyme MnSOD. I hypothesized that MC1R/cAMP increases the gene expression of MnSOD. I tested the role of MC1R in the induction of MnSOD protein levels and activity. Lastly, I explored the role of the MC1R in mitochondria metabolism as a potential functional effect of MC1R pathway. The MC1R/cAMP might be a potential target for the induction of antioxidant defenses after exposure to solar UV.

SPECIFIC AIM:

To study the role of MC1R in antioxidant defenses and mitochondrial oxidative phosphorylation.

a)   To explore the expression of oxidative stress related genes after pharmacologic induction of the MC1R pathway.

b)   To study a mechanism of levels/activity of MnSOD after activation of the MC1R/cAMP signaling pathway.

c)   To examine the role of MC1R/cAMP in mitochondria metabolism.

METHODS AND MATERIALS

CELL LINES AND PLASMIDS

A375, SKMel2 and HEK293 cell lines were purchased from ATCC. pcDNA3.1/MnSOD (human) 3`-FLAG-MnSOD was provided by Dr. Jian-Jian Li from the University of

California Davis. WT MC1R and mutated MC1R R160W were constructed with the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA.). Mouse primary melanocytes were isolated from C57BL/6 *extension K14-SCF* transgenic *extension*.

REAGENTS

The following antibodies were purchased commercially: SOD2 (1H6; western blot) (Pierce-Thermo Scientific, Waltham, MA.); GSR and GPX antibodies (Santa Cruz Biotechnology, Santa Cruz, CA.); GAPDH (Life Technologies, Grand Island, NY); VDAC, HSP70, FLAG (DKDDDDK),PKA P/S antibodies (Cell Signaling, Boston, MA.); HRP-conjugated secondary antibodies (Abcam, Cambridge, MA.) SOD determination kit (Sigma-Aldrich, St. Louis, MO.) and the Seahorse Analysis reagents (Seahorse Bioscience Massachusetts, USA.)

ISOLATION OF PRIMARY MELANOCYTES

The protocol used was published from our laboratory from the article of Scott and colleagues (Scott, Wakamatsu et al. 2009). The primary murine cells were obtained from $Mc1r^{e/e}$ $Tyr^{+/+}$ C57BL/6J animals unless otherwise stated. Melanocytes were selected for and grown in a Ham's F12 media and established normal murine keratinocyte conditioned media mixture.

MRNA ISOLATION FROM TISSUE SAMPLES

Skin biopsies were taken at indicated time-points. Skin tissues were ground in liquid nitrogen with a mortal and pestle. The skin was homogenized with Trizol® reagent following manufacturer protocol. The mRNA was processed using the RNAeasy kit (Qiagen, Valencia, CA.) following manufacturer's instruction. mRNA was quantified, normalized and converted to cDNA using the iScript cDNA kit (Qiagen, Valencia, CA.).

qPCR

The cells were plated in 35mm dishes and with 10µM forskolin or 0.1% ETOH. Untreated cells served as control samples. The cells were dissociated by treatment with 0.25% trypsin/EDTA at 37°C for 5 min. The cells were harvested, centrifuged and RNA was isolated using the RNAeasy kit (Qiagen, Valencia, CA.). RNA was converted to cDNA using the iScript cDNA kit (Qiagen, Valencia, CA.). The cDNA from mouse tissue was used to perform quantitative PCR assay by the LifeCycle Real-time PCR (Roche, Indianapolis, IN) with the β-actin as a reference gene included for each reaction. The levels of expression of genes in stimulated cells were compared with the untreated control groups. The cDNA from human melanocytes was used to perform quantitative PCR assay by the CF-96 Biorad qPCR with the 18S as a reference gene included for each reaction. The levels of expression of genes in stimulated cells were compared with the untreated control groups.

XL-PCR

Genomic DNA was extracted using a commercially available Qiagen genomic-tip kit (Qiagen, Inc., CA). The quantitative polymerase chain reaction (qPCR) assay measures the average oxidative lesion frequency on mtDNA. DNA damage was quantified by comparing the relative efficiency of the amplification of a 13.4kb mtDNA gene fragment and normalized to 250bp fragment (this small fragment has a statistically negligible chance of sustaining oxidative stress-induced base damage). PCR conditions used in this study were based on previously reported sequences for mtDNA primers (Ayala-Torres, Chen et al. 2000, Jarrett and Boulton 2005). qPCR was carried out on a DNA Engine thermal cycler with all reactions being a total volume of 100µl containing 15ng of total genomic DNA, 1unit of XL rTth polymerase, 3.3 XL PCR buffer II (containing potassium acetate, glycerol and DMSO) and final concentrations of 200µM dNTP`s, 1.2mM Mg(OAc)$_2$ and

0.1µM primers. The gene fragments were amplified using the following thermo-cycling profile: The PCR was initiated with the addition of the 1 unit of XL rTth polymerase when samples had reached a temperature of 75°C. This was followed by an initial penetration for 1 min at 94°C, cycles of denaturation at 94°C for 30s and primer *extension* at 60°C for 13 min. After the PCR cycles had been completed, a final *extension* at 72°C for 10 min was performed. The nuclear and mitochondrial gene products underwent 28 cycles and 26 cycles of thermo-cycling, respectively. After the completion of the qPCR, the gene products were resolved on a 1% agarose gel and digitally photographed on a UV transilluminator (UVi Tec, UK). The intensity of the PCR product bands was quantified with Scion Image analysis software (Scion Corporation, Version Beta 4.0.2).

CHLORAMPHENICOL RESISTANCE ASSAY

Chloramphenicol (CAP)-resistance was measured as a measurement of mtDNA mutagenesis. A375 cells were seeded onto 6-well dishes at 500 cells/well. After the 24 hours of incubation, the growth medium was replaced with medium containing 10uM Forskolin and incubate for 24 hrs. The cells were irradiated at a sub-lethal dose of UVB (5J) and incubate for 24 hours. If the experiment responded to a schedule of treatments, step 2 is repeated before each irradiation. At the end of all treatments, the cells were incubated with growth medium containing 1mM CAP. The cultures were maintained until colonies visible to the naked eye were formed; the colonies were then fixed with methanol and stained with 0.5% crystal violet in 50% methanol (roughly takes 2 weeks). The mutation frequency is calculated by dividing the existing colonies by the amount of cells plated.

WESTERN BLOT, IMMUNO-PRECIPITATION AND ACTIVITY ASSAY

Cell lines were lysed in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and 1X Halt Protease and Phosphatase Inhibitor

Cocktail, EDTA-Free (Thermo Scientific, Waltham, MA.)). Total protein was quantified using Pierce BCA protein assay (Thermo Scientific, Waltham, MA.). The amount of protein per sample was normalized and loaded onto SDS-PAGE gels. Immunoblotting was performed using antibody manufacturer protocols. Immuno-precipitations were performed with equal amount of total protein incubated overnight at 4°C with 5μg of antibody and protein G agarose, washed three times with RIPA buffer, and separated by SDS-PAGE prior to immunoblotting. For indicated experiments the SDS-PAGE gel was stained with Colloidal blue. Several bands were selected for their identification at the Proteomic facility of the University of Kentucky. All immunoblotting was performed with enhanced chemiluminescence (ECL) and quantified with the STORM system. Bands were quantified using Image J (NIH, Bethesda, MA.). Equal mitochondrial and whole cell protein were used for the SOD determination assays, and the assay was performed as previously described (MacMillan-Crow, Crow et al. 1996) and following manufacture's protocol (Cayman Chemical Co. Ann Harbor, MI.). Potassium cyanide (KCN) 1mM solution was used as an inhibitor of Cu/ZnSOD and ECSOD at indicated experiments.

MITOCHONDRIA ISOLATION

The cells were plated in 150mm dishes and treated for cAMP stimulation. Treatments included 10μM forskolin or 0.1% vehicle control and untreated cells as the control samples. The cells were dissociated by treatment with 0.25% trypsin/EDTA at 37°C for 5 min. The cells were harvested, centrifuged and mitochondria were isolated using a buffer containing 10mM NaCl, 1.5mM MgCl$_2$, 10mN Tris-HCl, pH 7.5. Cells were homogenized on ice and centrifuged. The sucrose gradient was prepared with a 2M sucrose solution in 10mM Tris-HCl and 1mM EDTA (TE buffer) following indicated dilutions. Pelleted material was layered on a 1:1 mixture of 1.7M sucrose and 1.0M sucrose and centrifuged at 24,000 rpm for 40 min. The fraction containing mitochondria was washed in TE buffer and

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centrifuged for 10 min at 13,000 rpm. The pellet (mitochondria) was re-suspended in 250nM sucrose. The solution was diluted in RIPA buffer.

TRANSFECTION FLAG-TAGGED MNSOD PLASMID

Designated cell lines were plated in 35mm dishes and transfected with 3`-FLAG-MnSOD, pcDNA3.1, $MC1R^{E/E}$ and $MC1R^{R160W}$ using Effectene Transfection Reagent (Qiagen, Valencia, CA.) according to manufacturer protocol.

PROTEIN KINASE ASSAY

PKA kinase assays were initiated by incubating 10nM recombinant catalytic subunit of PKA enzyme (Invitrogen, Grand Island, NY.) and HA-tagged MnSOD (Fitzgerald, Acton, MA.) at 30°C for 10 min, in 40mM Tris-Cl (pH 7.5), 10mM $MgCl_2$, 1mM DTT, 100µg/ml BSA, and 10µM ATP. The reaction was stopped by the addition of 10µl of 100mM EDTA. The extent of PKA phosphorylation was measured by immune-blot with anti-phosphoserine (Abcam Cambridge, MA.). HA-tagged MnSOD was immunoprecipitated with anti-HA overnight at 4°C and protein G agarose, washed five times with RIPA buffer, and separated by SDS-PAGE prior to immunoblotting. All immunoblotting was performed with enhanced chemiluminescence (ECL) and quantified with the STORM system.

SEAHORSE ANALYSIS

Seahorse analysis experiments were performed after cAMP stimulation for 1, 3, 6 and 24 hours with 10uM forskolin or 0.1% ETOH before exposure to UVB consisting of two Westinghouse F15T8UV-B lamps at 500, 1000, 1500 and 5000 J/cm^2. Oxygen-consumption rates (OCR) was analyzed at the University of Kentucky Free Radical Biology and Cancer Shared Facility. OCR was controlled at all times during the experiment to exclude any pathway independent effect. Fccp, an uncoupling agent, was used to accelerate the electron transport chain (ETC), and doses were optimized together with amount of cells per experiment. Then, the Seahorse system measured the OCR (ρmol)

and what impact cAMP stimulation has on oxidative phosphorylation. The OCR was measured in triplicate during maximal respiration controlled by fccp and in the presence of rotenone as an ETC inhibitor. Values were normalized to the protein concentration per sample, and OCR levels were compared between untreated vs cAMP-stimulated conditions.

STATISTICAL ANALYSIS

The data was analyzed between cohorts of replicate samples (n = 3) by one way ANOVA using the Bonferroni post-test (Graph Pad PRISM software). p values <0.05 were considered statistically significant.

RESULTS

MC1R INCREASES EXPRESSION OF ANTIOXIDANT ENZYMES AT THE TRANSCRIPTION LEVEL.

I explored the role of the MC1R as a regulator of the gene expression of several antioxidant enzymes. Although MC1R pathway has been well described as a potent regulator of melanin production and skin pigmentation, there are limited studies looking at its role in the expression of antioxidant enzymes in the skin. Mc1r$^{e/e}$ Tyr$^{+/+}$ *extension* mice were used (the same mice that showed rescue of melanin synthesis after forskolin treatment). I performed a kinetic experiment measuring mRNA expression of MnSOD, CAT, GPX and Nfe2l1 or NRF2 (Figure 3-2). There was a 2.5-fold increase for the expression of SOD2 after 6 hours of topical forskolin treatment. Gene expression of MITF was used as an internal control for the experiment. There was no significant difference for other antioxidant enzymes compared to the 0 hour time-point. An important caveat to consider was that mRNA was isolated from whole skin samples (dermis and epidermis) from which melanocytes constitute roughly 10 percent of the cells present. Since MC1R is located in the plasma membrane of melanocytes, I isolated primary melanocytes from Mc1r$^{e/e}$ Tyr$^{+/+}$ mice. I tested the expression of MITF in these primary cells and there was

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an increase in MITF gene expression 3, 6 and 24 hours after forskolin treatment (Figure 3-3A). α-MSH was included in this experiment as a negative control in these MC1R-defective cells, and there was no induction of MITF after α-MSH treatment. Gene expression of SOD2 nor NFE2l2 (NRF2) increased after FSK treatment, suggesting that perhaps SOD2 and NRF2 are not target of the transcription factor MITF in mouse melanocytes. Then, human melanoma cell lines with mutated MC1R R151C were tested for the expression of MITF after forskolin treatment in several time points (Figure 3-3B). There was an increase of MITF expression in a time dependent matter with the higher expression at 6 hours. I selected the 6 hour time point to analyze a gene array that included antioxidant enzymes, several transcription factors and other protein regulators by qPCR. An oxidative stress panel for 60 genes was tested in response to MC1R activation. Out of the genes tested, 22 showed a positive response (at least two-fold increase). Of these, FOXO1, BCL2, JUN, MAPK1, 3 &9, STAT1, HSPD1 & DDIT3 were induced. These genes are known to have a role in the regulation of apoptosis. The TLR4 is a Toll-like receptor was increased together with the inflammatory cytokine IL1B. The activation of the adaptive immune system is considered a protective effect in the skin since it promotes healing in the skin after UV-induced sunburn (Greene, Hoover et al. 1978). Moreover, there was an increase in the expression of the antioxidant enzymes SOD1, GPX1, & 3, Nrf2 and NF-κB. Both Nrf2 and NF-κB were upregulated to a 6 and 4-fold increase, respectively. RELA is a subunit of NF-κB and its expression was increase as well. Interestingly, some downstream targets of Nrf2 and NF-κB did not show a significant effect after MC1R activation by forskolin in human melanocytes. For example SOD1, 2 & 3 were down-regulated and they are known to be activated by NF-κB. Nrf2 regulates the expression of GSTP1 and GCLC. Both are involved in the glutathione pathway and their gene expression after the 6-hour time point was down regulated. Additional kinetics experiments are require to address the effect on MC1R on these genes. There was no

increase in the expression of several antioxidant enzymes (CAT, TP53, MDM2 among other genes relative to the expression of GAPDH) (Table 3-1), but there were significant increases in the expression of PRDX3 and MAPK9 (Table 3-1). Although some genes were down regulated, MC1R activation had a role in the up-regulation of several regulators of antioxidant enzymes and oxidative stress related genes at the transcriptional level.

MC1R INCREASES MNSOD PROTEIN LEVELS IN MITOCHONDRIA AT EARLY TIME POINTS.

I wanted to study the role of MC1R in the protein levels of MnSOD in melanocytes. There was no significant increase in the protein levels of MnSOD (Figure 3-4A), from 1 to 24 hours after cAMP stimulation in whole cell lysate. The protein levels of GSR and GPX (Figure 3-4B) did not increase after a treatment of 6 hours. However, there was an increase in the protein levels of MnSOD in mitochondria lysate at early time points (Figure 3-4C, D). Furthermore, there was an increase in MnSOD protein levels after cAMP stimulation plus 50J UVB or 50uM of H_2O_2 in mitochondria isolated from MC1R R151C melanocytes (Figure 3-5) after 1 hour of forskolin treatment. I transfected human embryonic kidney cells (HEK293) with wild-type and mutated MC1R to test for the protein levels of MnSOD. There was a significant increase in the levels of MnSOD in MC1R R160W with forskolin (Figure 3-6). However, HEK 293 transfected with wild-type MC1R showed no induction of the protein levels of MnSOD. These results at early time points suggesting that the up-regulation of MnSOD protein levels may not involve transcriptional up-regulation but rather MnSOD transport and/or stability.

MC1R SIGNALING INCREASES MNSOD INTERACTION WITH CHAPERONE PROTEIN HSP70.

To investigate the possibility that cAMP-mediated increases in MnSOD were regulated by binding partners, I made used of FLAG tagged-MnSOD constructs obtained from Dr. Jian-Jian Li from the Comprehensive Cancer Center at UC Davis. I tested MC1R R151C cells after 10uM forskolin and 50uM H_2O_2 treatment for 1 hour. Cellular lysates were

immunoprecipitated for FLAG and analyzed by western blot. The SDS-PAGE gel was stained with colloidal blue and treatments were compared. The bands that increased or decreased by forskolin treatment were selected and removed for the gel to be sent for analysis. A band close to the 70 kDa size was sent for identification with the proteomic facility (Figure 3-7A). They identified it to be heat shock protein 70 (HSP70) increased in associated with FLAG-tagged MnSOD after forskolin treatment. I tested the cell line with forskolin followed by 30 minutes of hydrogen peroxide and found an increase in the interaction of HSP70 with FLAG after treatment with forskolin (Figure 3-7B, C). HSP70 was also increase in the western blot for whole protein without IP. However, when the cells were treated with H_2O_2 the whole cell increase of HSP70 did not change. But, the interaction remained in the IP FLAG-tagged MnSOD after H_2O_2 treatment. HSP70 is a chaperone protein that is found in the cytoplasm. As a chaperone, it has a role of transporting proteins around the cell and HSP70 is linked to translocation of glutathione to the mitochondria (Guo, Wharton et al. 2007).

MNSOD INTERACTION WITH HSP70 IS PKA DEPENDENT.

I wanted to test if PKA had a role in the increase of HSP70 interacting with FLAG tagged-MnSOD. I tested the inhibitor H89 that blocks the PKA catalytic subunit (Figure 3-8A). I treated FLAG tagged-MnSOD A375 (MC1R R151C) cell line for one hour with H89. Then, the cells were treated with 0.1% ETOH and 10uM forskolin for 1 hour. I saw a decrease in the interaction between HSP70 and FLAG. I tested doses of H_2O_2 and chose 50uM for 30 minutes (Figure 3-8B). I observed no increase in HSP70 after I.P. with FLAG after adding H89. The protein sequence of MnSOD identified two possible PKA phosphorylation sites potentially explaining cAMP-enhanced interactions with HSP70. These sites could be used as target in future studies for site-directed mutagenesis to continue further studies about HSP70 interaction with MnSOD

FORSKOLIN RESCUE OF MC1R SIGNALING INCREASES MnSOD ACTIVITY.

I wanted to investigate whether the protein levels of MnSOD and its interaction with HSP70 might result in an increase in MnSOD protein activity. I tested weather cAMP affects the activity of MnSOD using the Superoxide Dismutase Assay Kit (Cayman Chemical Co., Ann Harbor, MI). Total protein (whole cell) was isolated from MC1R R151C cell lines. Efficacy of superoxide removal using xanthine oxidase as a standard for ROS production (Figure 3-9) was quantified. There was an increase in the levels of superoxide dismutase activity after 1 and 3 hours of forskolin treatment in whole cell lysate (Figure 3-9A). These results include the activity of Cu/ZnSOD, MnSOD and ECSOD. Potassium cyanide (KCN) was added to the protein solution to inhibit the activity of Cu/ZnSOD and ECSOD. The inhibition of Cu/ZnSOD and ECSOD enable the detection of MnSOD activity exclusively. Figure 3-9A shows no significant difference in MnSOD activity between each time point in whole cell protein lysate. The concentration of xanthine oxidase was decreased within minutes of the reaction and there was no difference between forskolin and vehicle. However, whole cell lysate treated with KCN showed a significant decrease in the presence of forskolin. Interestingly, there was an increase in MnSOD activity (U/mg) at the one hour time point (Figure 3-9B). Then, mitochondria protein lysate was tested together with potassium cyanide to measure MnSOD activity. Similarly, there was an increase at the one hour time point after forskolin treatment. Therefore, I concluded that the activation of MC1R/cAMP signaling increased MnSOD activity at early time points.

MC1R SIGNALING DOES NOT AFFECT MITOCHONDRIA METABOLISM.

MnSOD is an antioxidant enzyme located in the mitochondria. I wanted to investigate if there was any effect in mitochondria metabolism after forskolin treatment. Seahorse analysis was optimized to test the oxygen consumption rate (OCR) after stimulation of cAMP in MC1R R151C cells (Figure 3-10). I tested the effects of one hour treatment in

order to compare the results with the protein levels and immune-precipitation assay. The results are shown as OCR percentage (A) and OCR concentration at ρMol/min. There was no increase in the OCR after cAMP stimulation at the basal metabolism, ATP production or maximal respiration. I included a dose of UVB irradiation of 50J to test effect UV as a positive control and investigated a possible protective effect after forskolin treatment. There was no significant difference between forskolin plus UVB and UVB alone within the same phase of mitochondria metabolism. However, there was a significant difference in the levels of maximal respiration comparing forskolin plus UVB with UVB alone.

A
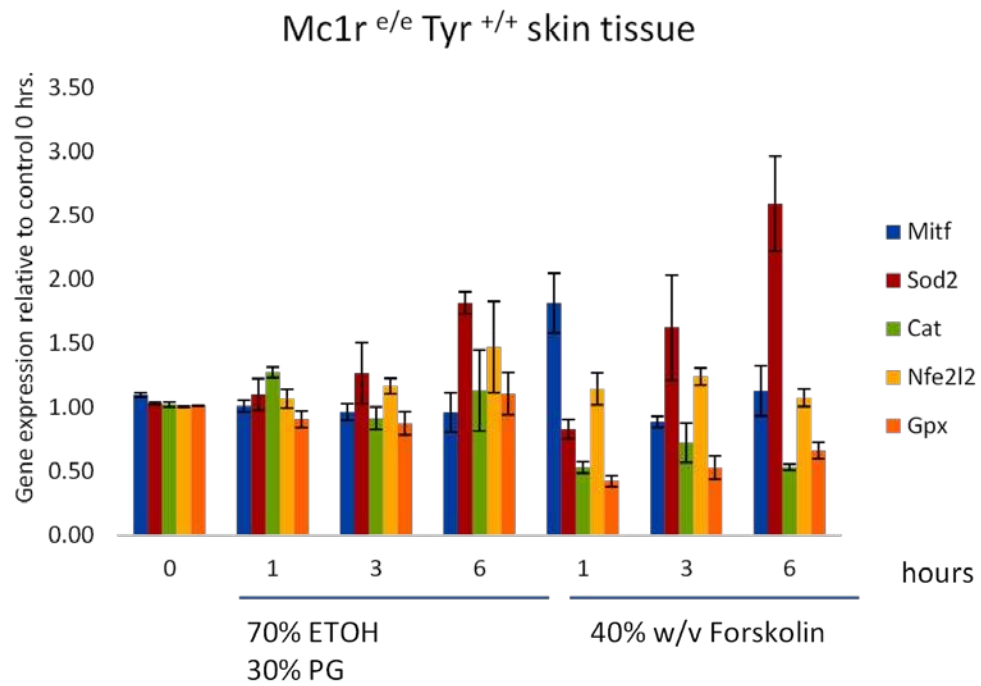
Figure 3-2. MITF and SOD2 gene expression were rescued after forskolin treatment in whole skin tissue.

Mc1r $^{e/e}$ Tyr $^{+/+}$ were treated with 40%w/v Forskolin and vehicle control (70% ETOH, 30% PG) for 1 to 6 hours. Skin biopsies were taken and mRNA was isolated and analyzed by qPCR using primers specific for mouse mRNA. The gene amplification was normalized to 18S and the relative amplification levels at all-time points after forskolin and vehicle treatment were normalized to 0 hours control.

A
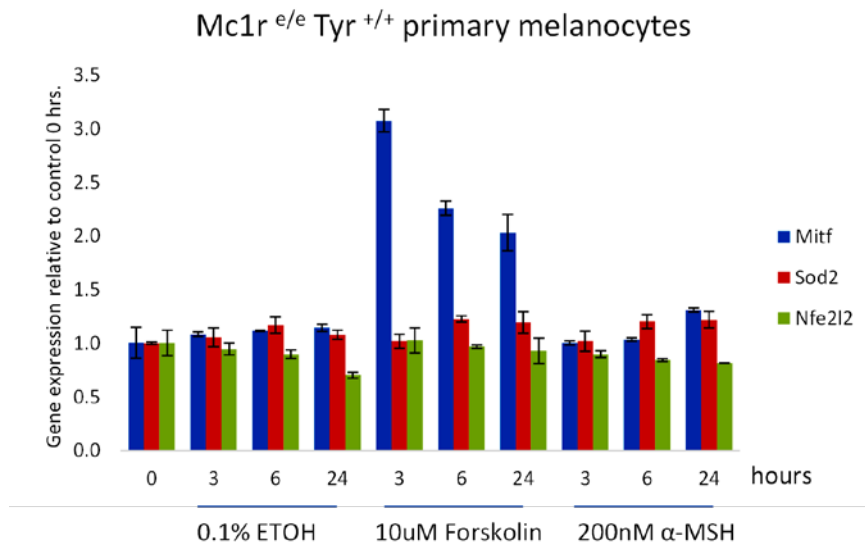
B
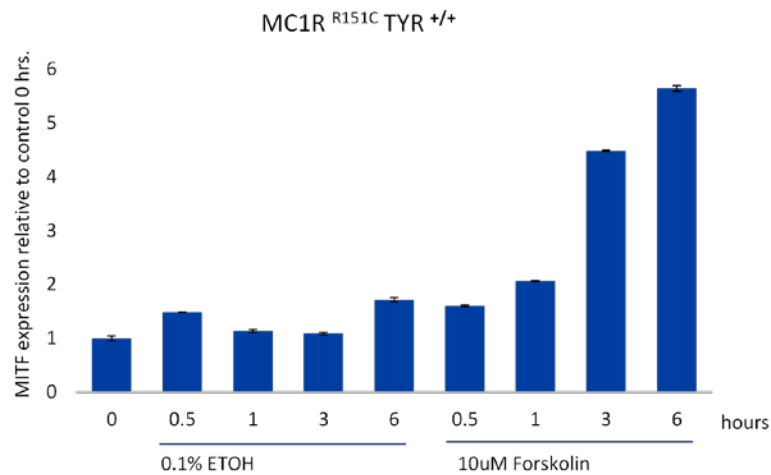
Figure 3-3. MITF gene expression was rescued after forskolin treatment.

(A) Mc1r $^{e/e}$ Tyr $^{+/+}$ primary melanocytes were treated with 10uM Forskolin and 0.1% ETOH for 3 to 24 hours. mRNA was isolated using RNAeasy kit (qiagen). The samples were analyzed by qPCR using primer specific for mouse mRNA. (B) Human melanocytes MC1R R151C were treated with 10uM Forskolin and 0.1% ETOH for 0.5 to 6 hours. mRNA was isolated and analyzed with qPCR using primer specific for human mRNA. The gene amplification was normalized to 18S (A) or GAPDH (B) and the relative amplification levels at all time-points after forskolin and vehicle treatment were normalized to 0 hours control.

Gene name	Fold change	Gene name	Fold change	Gene name	Fold change
GAPDH	1.000	PARKAA2	1.905	SOD2	0.586
GPX3	7.945	SOD1	1.705	LCN2	0.547
FOXO1	6.589	JAK2	1.659	SHC1	0.547
BCL2	6.320	APP	1.591	GCLM	0.521
NFE2L2	5.938	CYP2E1	1.464	PRDX1	0.497
STAT1	5.696	PRKCZ	1.454	SERPINE1	0.451
MAPK1	5.352	CAT	1.434	DHCR24	0.444
NFKB1	4.659	GLRX2	1.424	TP53	0.429
GPX1	4.563	MDM2	1.395	G6PD	0.412
LDHA	4.500	PRDX5	1.395	PRDX4	0.376
JUN	4.347	GPX4	1.266	GCLC	0.361
MAPK8	4.028	GSR	1.157	NQO1	0.275
PRDX3	3.918	PON2	1.157	EGFR	0.268
RELA	3.160	PTGS2	1.149	AC0088.01	0.245
DDIT3	2.990	AKT1	1.000	PRDX6	0.187
HSPD1	2.868	TXNRD1	1.000	APEX1	0.150
TLR4	2.657	HPRT1	0.807	PRDX2	0.115
MAPK9	2.144	GSTP1	0.801	HMOX1	0.103
PRKCD	2.114	OGG1	0.737	APOA1	0.100
MIF	2.085	CASP3	0.717	CD36	0.100
SIRT1	2.071	PRKCA	0.683	IL6	0.100
ILI1B	2.028	MAP3K5	0.678	PRKCB	0.100
SRC	2.014	ADIPOQ	0.646	CYBA	0.035
		CLU	0.646	SOD3	0.000

Table 3-1. Antioxidant enzymes gene array expression after forskolin treatment.

Human melanocytes (MC1R R151C) were treated with 10uM Forskolin and 0.1% ETOH for 6 hours. mRNA was isolated and analyzed with Primer PCR Assay for oxidative stress pathway (Biorad, Hercules CA.) using primer specific for human mRNA. The gene amplification was normalized to GAPDH and the relative amplification levels after forskolin treatment were normalized to vehicle control (0.1% ETOH).

A

MC1R R151C

FSK	-	-	-	-	-	+	+	+	+	+
hours	0.5	1	3	6	24	0.5	1	3	6	24

MnSOD

| | 1.0 | 0.6 | 0.6 | 0.7 | 0.8 | 1.0 | 0.8 | 0.8 | 0.9 | 0.9 |

GAPDH

Whole cell

B

MC1R R151C

FSK	-	+	-	+
H_2O_2	-	-	+	+

GSR

| | 1 | 0.9 | 0.6 | 0.5 |

GPX

| | 1 | 0.5 | 0.2 | 0.2 |

GAPDH

Whole cell

C

MC1R wild-type

FSK	-	-	+	+
UVB	-	+	-	+

MnSOD

| | 1.0 | 1.3 | 2.0 | 2.0 |

VDAC

Mitochondria

D

MC1R R151C

FSK	-	-	-	+	+	+
hours	1	3	6	1	3	6

MnSOD

| | 1.0 | 0.8 | 0.6 | 1.4 | 1.5 | 1.9 |

VDAC

GAPDH
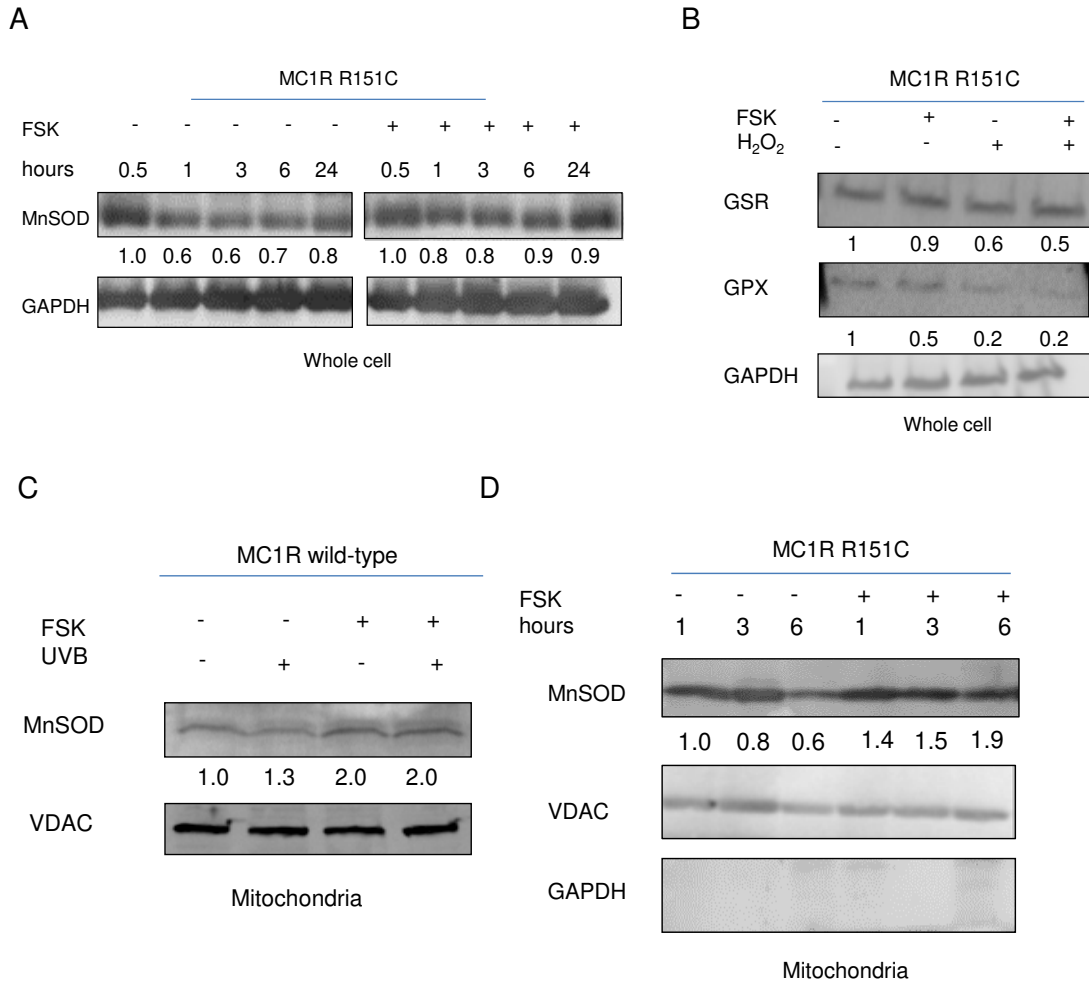
Mitochondria

Figure 3-4. MnSOD protein expression did not change after forskolin treatment in whole cell lysates but did increase in mitochondria.

Human melanocytes MC1R R151C were treated with 10uM Forskolin and 0.1% ETOH for 30 minutes to 24 hours. Whole cell (A, B) and mitochondria (C, D) protein was isolated and quantified to be examined by western blot. (B) The cells were treated for 6 hours with or without 50uM H_2O_2. (C) The cells were treated for 30 min. with 10uM forskolin and 50J of UVB. (D) The cells were treated for different time-points with 10uM forskolin and 0.1% ETOH. The relative expression of MnSOD (relative to loading control GAPDH (cytoplasm) or VDAC (mitochondria)) was graphed after forskolin treatments were normalized to initial time-point.

Panel C was performed by Dr. Stuart Jarrett, PhD.

A
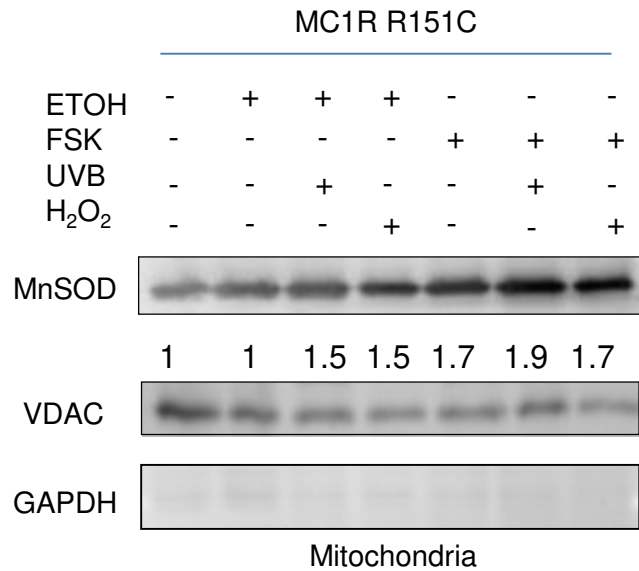
Figure 3-5. MnSOD protein expression increased after forskolin treatment in mitochondria.

Human melanocytes MC1R R151C were treated with 10uM Forskolin, 0.1% ETOH for 1 hour, 50 J UVB and 50uM H_2O_2. Mitochondria protein was isolated using sucrose gradient and quantified to be examined by western blot. The relative MnSOD expression (relative to loading control VDAC) was graphed after forskolin treatment were normalized to vehicle control.

A
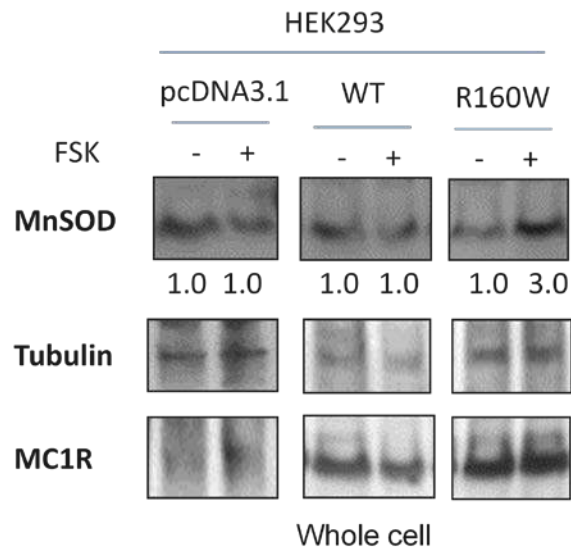
Figure 3-6. The role of mutated and wild-type MC1R signaling role in the expression of MnSOD.

HEK 293 were transfected with wild-type MC1R and R160W. The transfected cells were treated for 1 hour with 10uM forskolin. Whole cell lysates were isolated, quantified by western blot against indicated-proteins.
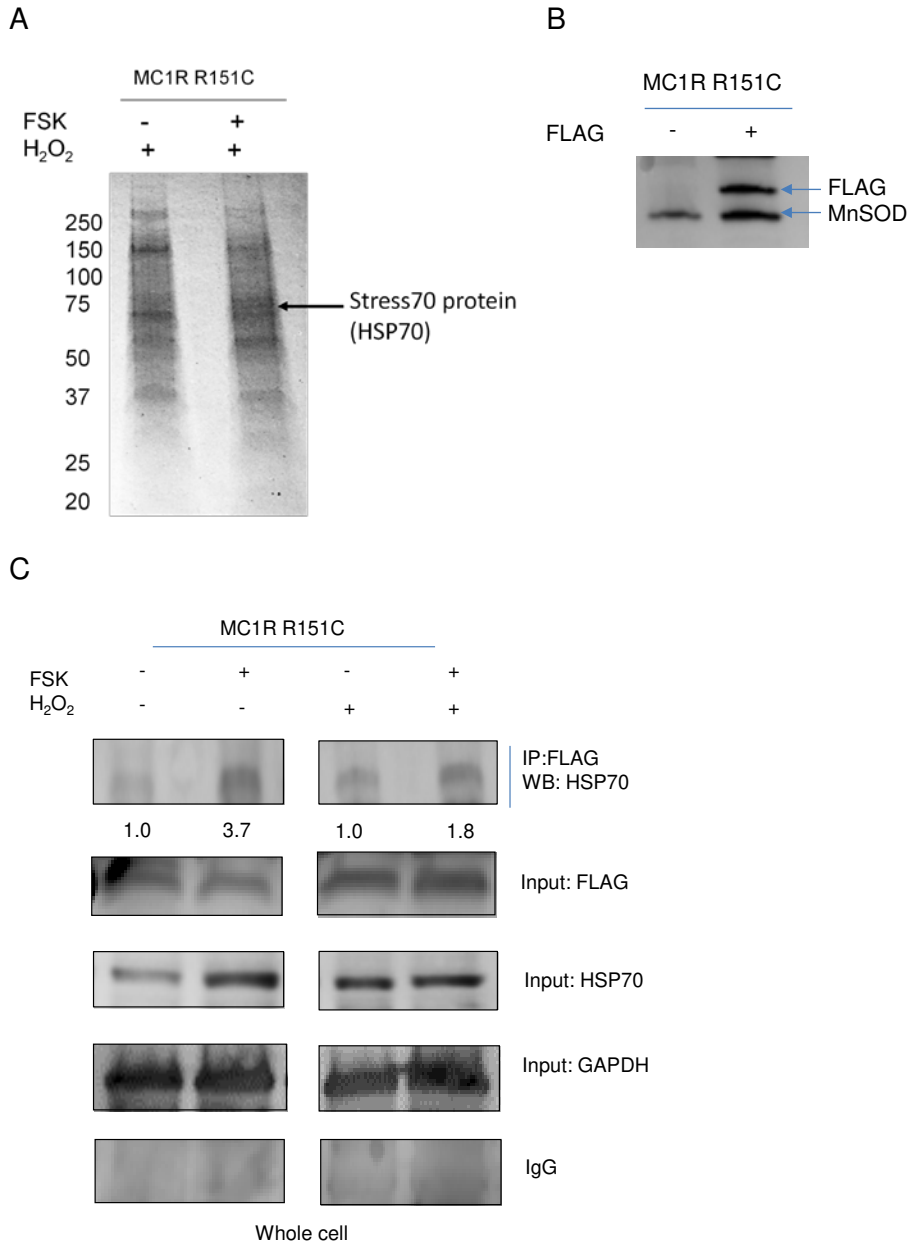
Figure 3-7. HSP70 binds to MnSOD after MC1R activation.

(A) Human melanoma MC1R R151C cell line were treated for 1 hour with 10uM forskolin or 0.1%ETOH and protein was isolated and IP of FLAG tagged-MnSOD was performed. Colloidal blue stain was performed and the indicated band was sent for proteomic identification. (B) Human melanoma MC1R R151C cell line transfected with FLAG tagged-MnSOD. Then, (C) the cells were treated with 0.1%ETOH and 10uM forskolin from 1 hour. 50uM H_2O_2 was added to the indicated samples for 30 min. Protein was isolated and IP of FLAG tagged-MnSOD was perform followed by a western blot for HSP70. (C) Whole cell protein (without IP) was included and a western blot was performed for the loading control FLAG.

A

MC1R R151C

FSK	-	+	-	+
H_2O_2	-	-	+	+
H89	+	+	+	+

IP:FLAG
WB: HSP70

1.0	0.8	1.0	1.2

Input: FLAG

Whole cell

B

Recombinant protein

PKA	+	+	+
MnSOD	+	+	+
ATP	-	+	+
H-89	-	-	+

Phospho Serine
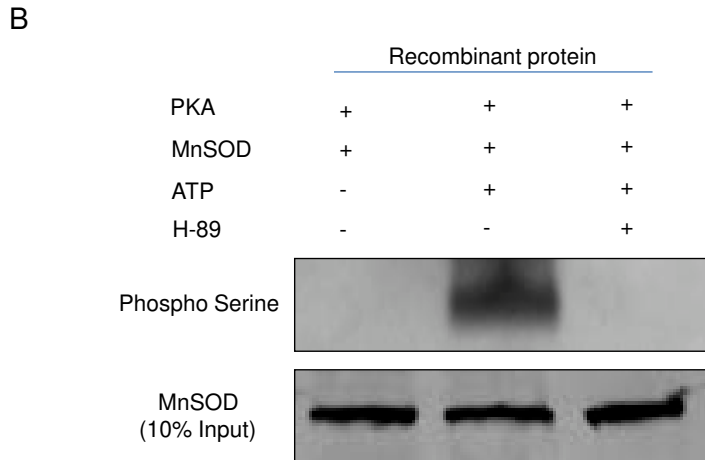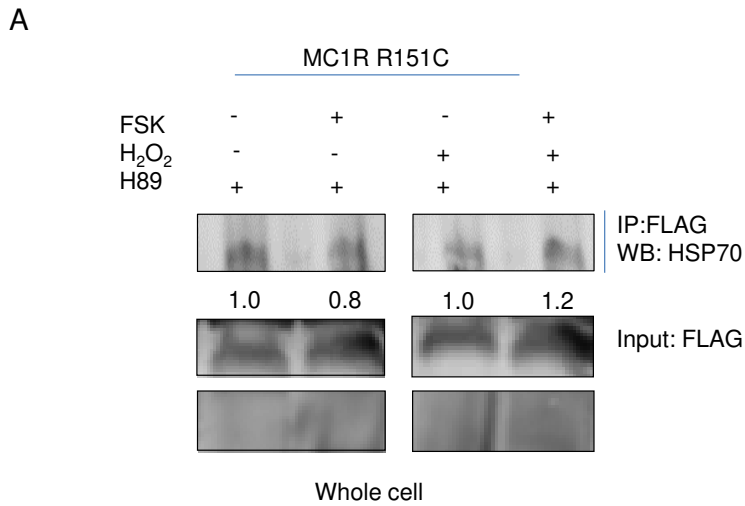
MnSOD
(10% Input)

Figure 3-8. FLAG-tagged MnSOD binding to HSP70 is PKA dependent.

(A) Human melanoma MC1R R151C cell line transfected with FLAG tagged-MnSOD treated with 10uM H89 for 1 hour. Then the cells were treated with 0.1% ETOH, 10uM Forskolin for an additional 1 hour. 50uM H_2O_2 was added to the indicated samples for 30 min. Protein was isolated and IP of FLAG tagged-MnSOD was perform followed by a western blot for HSP70. Input protein (without IP) was included and a western blot was performed for loading control FLAG or MnSOD.
(B) The PKA kinase assay was performed using recombinant protein HA-MnSOD and PKA. Both were mixed for 10 minutes and HA-MnSOD was IP and separated in a SDS-PAGE. Immunoblotting was performed for p-serine.

Panel B was performed by Dr. Stuart Jarrett, PhD.

A

B

C
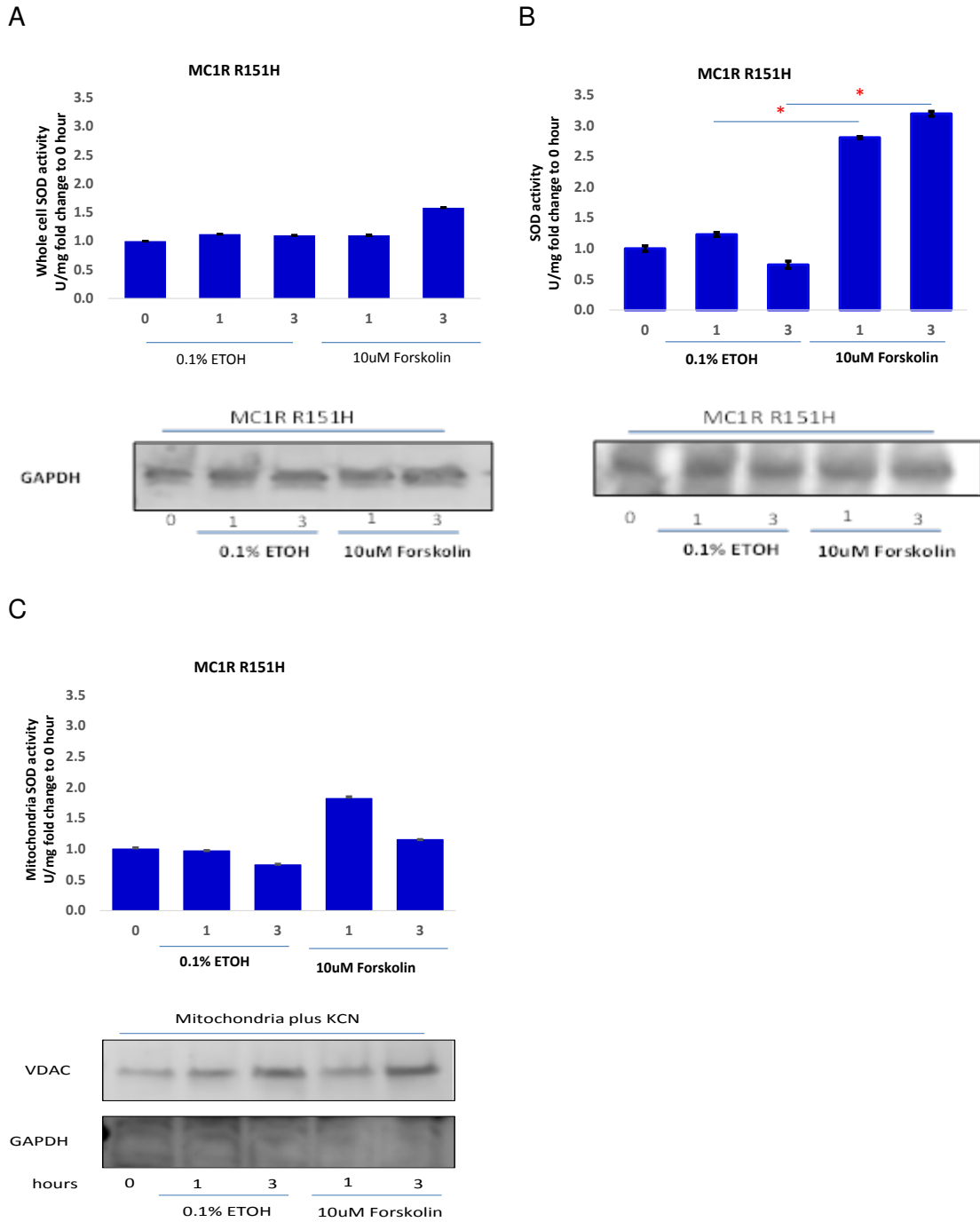
Figure 3-9. MnSOD protein activity does not change after forskolin treatment. Human $MC1R^{R151C}$ melanocytes were treated with 10uM Forskolin and 0.1% ETOH for 1 to 3 hours. (A) Whole cell protein, (B) whole cell protein plus 1mM potassium cyanide (KCN) (C) and mitochondria protein plus KCN were isolated and quantified to be examined by SOD activity assay (Cayman Chemical Co., Ann Harbor, MI.). The activity of SOD in U/mg was graphed relative to 0 hour control sample. GAPDH was the loading control for whole cell lysate and VDAC for mitochondria lysate. * $p < 0.01$

A
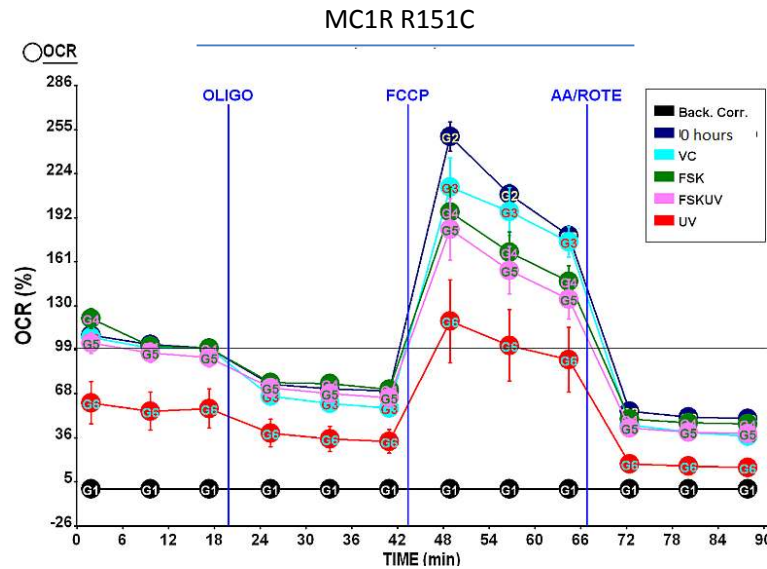
B
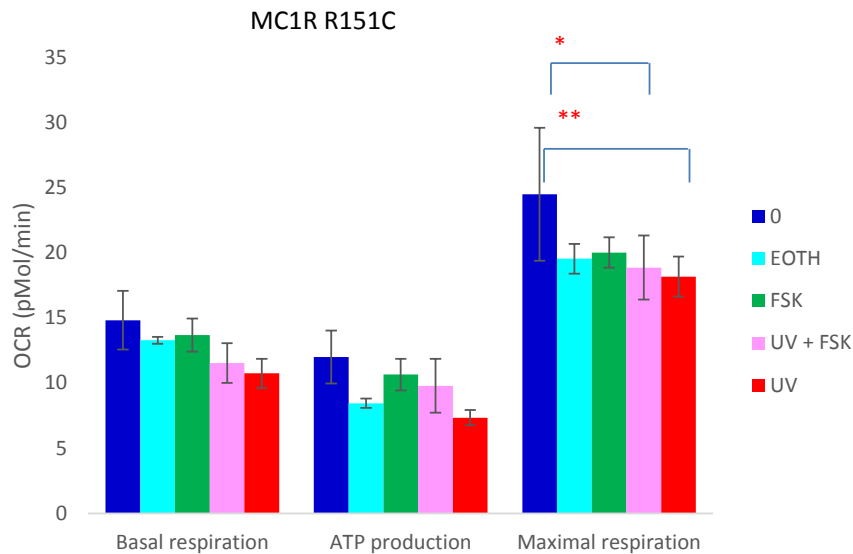
Figure 3-10. MC1R signaling did not affect mitochondria metabolism.

(A) The percentage and (B) pmol/min concentration of the oxygen consumption rate (OCR) was measure using the Seahorse Analysis. Human melanocytes MC1R R151C were treated with 10uM Forskolin, 0.1% ETOH for 1 hour, and 50J UVB. After initial treatments, the cells were treated with Oligomyocin A, it is an inhibitor of ATP synthase. FCCP- uncoupled respiration, measure ETC integrity, functionality and capacity. AA/Rotenone- inhibits mitochondria NADPH dehydrogenase/complex 1 to test for the pathway of mitochondria metabolism using the Seahorse analysis. The OCR was quantified per each treatment. The OCR ± SD results are reported as pmol/min, * $p \leq 0.05$ ** $p \leq 0.01$.

DISCUSSION

In this chapter, I hypothesized that MC1R signaling pathway regulates the expression of antioxidant enzymes. The gene expression in whole skin samples might be different than purified melanocytes since the skin harbors many different types of cells. Forskolin is able to rescue MC1R signaling in-vivo, therefore, I took skin biopsies of the Mc1r$^{e/e}$ Tyr$^{+/+}$ mice treated at different time points with forskolin. There was an increase of 2.5 fold of SOD2 gene expression in the skin samples. However, SOD2 did not show an increase after isolating primary melanocytes from Mc1r$^{e/e}$ Tyr$^{+/+}$ mice. The presence of other cell lines in the skin might have a role in the up-regulation of SOD2. It is known that NF-κB is involved in the immune system and mediates the transcription of SOD2. It is possible that when melanocytes are isolated from the skin, they lose the interactions with specific cell types that promote gene expression of SOD in the skin.

Here, I report a protective role of MC1R in the expression of transcription factors, post-translational regulators and proteins that are part of the oxidative stress pathway. The 6 hour time point had its higher expression of the transcription factor MITF, a downstream target of MC1R pathway. Interestingly, genes involved in the base excision repair pathway such as OGG1 and APEX1 showed no increase at the gene expression. However, there was a significant increase in the expression of NF-κB, which regulated the expression of SODs, and an increase in Nrf2 that regulates the expression of glutathione and glutathione regulating proteins such as GPX1 and GPX3. A 6 hour time point provides limited information about the mechanism of action by which MC1R increases the protein levels of these enzymes. However, in support of our hypothesis, it is clear that several antioxidant enzymes may be up-regulated at the gene level after forskolin induced MC1R signaling rescue.

SOD2 was not increased after cAMP production in human melanoma cell line compared to results in mouse melanocytes. Therefore, I hypothesized that there might be

up-regulation MnSOD at protein levels in MC1R-deficient human melanocytes after forskolin treatment. Remarkably, MC1R pathway regulated the protein levels of MnSOD in mitochondria, suggesting a role at the post-translation level since an early time point (30 minutes) seems inadequate for a transcriptional effect. However, there was a decrease in the level of GSR and GPX protein levels after the addition of H_2O_2. This is not surprising since GSR and GPX are the first line of antioxidant defense after oxidative injury. A decrease in their protein levels leads to the activation of other antioxidant enzymes and their regulators. MnSOD is only expressed in the mitochondria, perhaps explaining why I was not able to observe cAMP-induced differences in whole cell lysate from human melanocytes. Rather, protein levels of MnSOD were increased only when mitochondria lysate was isolated.

The transfection of wild-type MC1R in HEK293 cells showed a slight decrease in the protein levels of MnSOD after MC1R activation with forskolin. Since HEK293 cell line is not skin specific, it is possible that MnSOD protein levels are regulated differently than in native melanocytes. One option to clarify this issue would be to inhibit the expression of MC1R with siRNA in human melanocytes and test for the protein levels of MnSOD before and after forskolin treatment. Other possibilities to explain high levels of MnSOD in unstimulated MC1R wild-type melanocytes include ligand-independent MC1R signaling or altered number of MC1R proteins expressed in the plasma membrane. HSP70 is known to have a role in the translocation of glutathione to the mitochondria (Guo, Wharton et al. 2007). Surprisingly, this protein interacts with MnSOD after the activation of MC1R signaling plus H_2O_2. MC1R deficient human melanocytes that were treated with forskolin that showed a 3.7 fold increase in MnSOD-associated HSP70 protein levels. However, there was no difference once H_2O_2 was added to the cells. Interestingly, immune-precipitation experiments using FLAG-tagged MnSOD showed an interaction between MnSOD and HSP70 after forskolin treatment and forskolin plus H_2O_2. This means that the

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MC1R signaling enhancing the interaction of MnSOD with HSP70 might have a role in the translocation of MnSOD to the mitochondria. This interaction was lost after adding a PKA inhibitor. This suggests that the mechanism of this interaction was PKA dependent. Based in the results on the protein kinase assay, there may be phosphorylation of MnSOD by PKA at any serine in MnSOD sequence. However, PKA might also have a role in the regulation of HSP70 as a transporter of MnSOD. Further experiments with recombinant protein might help to decipher a mechanism. Future experiments would focus on the identification of specific phosphorylation site(s), mutating them and determining effects on interactions between MnSOD and HSP70. The study of a possible phosphorylation of MnSOD increasing its interaction with the chaperone protein HSP70 is a potentially interesting discovery. However, it is important to consider that the regulation of the phosphorylation network in the cell requires both kinases and phosphatases and therefore may be more complex than one single post-translational modification. Although we focused on PKA since it is a target of cAMP, there are phosphatases that might be target of cAMP such as protein phosphatase 2A (PPA) which is inhibited by cAMP (Dodge-Kafka, Bauman et al. 2010) and opens an interesting mechanism of phosphatase regulation by cAMP that might be relevant in melanocytes.

I hypothesized that the activity of MnSOD was up-regulated after forskolin treatment in MC1R deficient human cell lines. There was an increase in the removal of superoxide radical generated by xanthine oxidase after forskolin induced MC1R signaling in MC1R deficient human melanocytes. These data are consistent with the increase in the expression of MnSOD in mitochondria after 1 hour of forskolin treatment. Based on these results, I showed for the first time that MC1R signaling might have a potential role in the up-regulation of MnSOD activity at early time points.

Moreover, there is a significant increase in maximal respiration comparing UVB treated cells with UVB plus forskolin-induced MC1R. UVB alone produces a significant

decrease in maximal respiration and forskolin seems to rescue that decrease. The analysis of mitochondria phosphorylation is challenging in epidermal melanocytes. This cell type contains lower levels of mitochondria compared with a muscle cell. Therefore, the values of this assay were lower than expected at normal rate of mitochondrial phosphorylation. Nonetheless, based in these results, there is a possible rescue in maximal respiration after forskolin treatment with or without UVB.

In this chapter, I demonstrated the role of MC1R/cAMP provides protection against DNA mutagenesis. In addition, MC1R enhances MnSOD levels/activity in mitochondria and does not affect the mitochondria metabolism as a potential functional effect of the pathway. MC1R signaling is a potential target for the prevention of UV-induced oxidative damage in the skin.

CHAPTER 4

SUMMARY

Pharmacologic activation of the MC1R signaling pathway protects the skin against UV-induced damage and promotes the activity of the antioxidant enzyme MnSOD. Individuals with deficiencies in the MC1R signaling pathway can benefit from pharmacologically-induce melanin production and rescue of MC1R signaling. Dark melanin is produced in the skin as a protective barrier. The effect is not only seen in UV-sensitive Caucasians but also on low-risk individuals of dark skin complexion. The pharmacologic stimulation of MC1R could be used as a therapeutic target to darken the skin and protect it against UV damage. The use of a cAMP stimulator in addition to sunscreen might contribute to a decrease in the incidence of melanoma.

I demonstrated that the activation of MC1R induces several epidermal defenses that protect the skin against oxidative stress by upregulating antioxidant defenses. Topical application of forskolin, can directly rescue downstream targets of the MC1R. Interestingly, I was able to see that by a second application of forskolin, the skin showed an accumulation of protective melanin, and melanin stayed in the skin as long as topical forskolin treatment were continued. Taking advantage of our mouse model, I studied the activation of the MC1R as a protective target against damaging UV doses. MC1R signaling enhanced antioxidant enzymes that provide protection against oxidative stress. Nrf2 is a key transcription factor in the regulation of antioxidant enzymes. MC1R increased the expression of Nrf2 together with several regulators of glutathione activity. I found an increase in the level of antioxidant enzymes expression after six (6) hours of cAMP stimulation in human melanocytes. In addition, the rescue of MC1R by forskolin resulted in an increase of MnSOD translocation to the mitochondria and higher MnSOD activity.

I studied possible mechanisms of action for cAMP induction of MnSOD. HSP70 is a chaperone protein that was found to bind to MnSOD. I observed interactions between MnSOD and HSP70 early after treatment with forskolin. Their interaction was lost when PKA inhibitor H89 was included. Thus, PKA might have a role by phosphorylating MnSOD to be recognized and translocated to the mitochondria. Clearly, cAMP-enhanced MnSOD is PKA-dependent. Figure 4-1 summaries the scope of chapter 2 and 3.
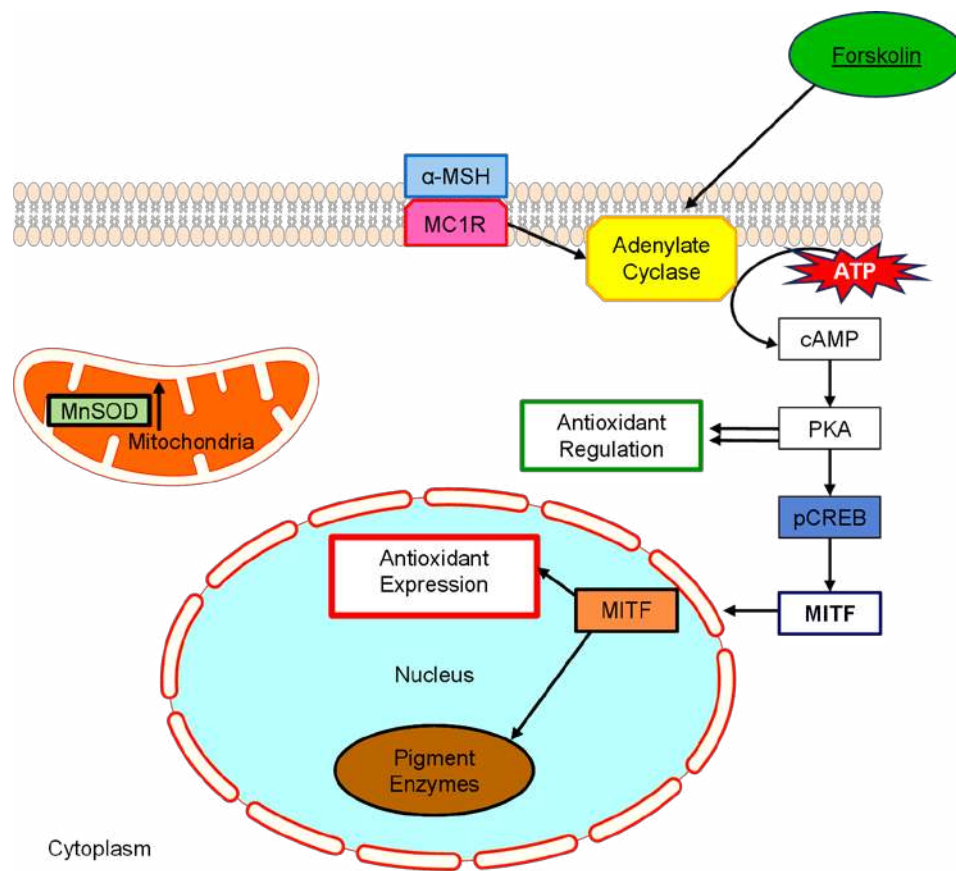
Figure 4-1 Schematic representation of the research project.

FUTURE DIRECTIONS

Studies link antioxidant defenses to many beneficial effects. Many synthetic and natural products have been reported to enhance levels of antioxidant enzymes, to prevent aging symptoms and to target UV-mediated damage. In addition, there are non-enzymatic antioxidants present in the skin. Some products that have the potential to induce antioxidant defenses in the skin include α-tocopherol, selenium, phloretin, ferulic acid, flavangenol, lipoic acid, and uric acid. I focused in the use of forskolin, a natural product that had been proved to be harmless as a topical treatment and now there is additional evidence that confirms its role in the rescue of MC1R and the induction of the protein levels of the antioxidant enzyme MnSOD.

Additional experiments are needed to determine the mechanisms by which of MC1R increases MnSOD levels in mitochondria. The proximity ligation assay (PLA) allows the study of two protein within 11nm proximity. Using this technique, one can to determine the location of the interaction between MnSOD and HSP70. I reported the inhibition of this interaction by adding H89. In addition, if the site of phosphorylation is identified, a site-directed mutagenesis assay can be performed for MnSOD abolishing of the interaction similar to the use of a PKA inhibitor.

Stimulating cAMP levels in the skin is attractive for a variety of reasons: Increasing cAMP levels in epidermal melanocytes stimulates pigment synthesis and cutaneous deposition of melanin. A proof-of-concept study using forskolin has already been shown in an animal model of the MC1R-deficient human (D'Orazio, Nobuhisa et al. 2006). The pharmacologic cAMP induction is not mutagenic (as opposed to UV). The use of spray tanning might be a safe alternative to the use of tanning beds. Sunless tanning would lessen the need for purposeful UV-seeking behavior among individuals desiring a darker skin complexion and would thus reduce the frequency of melanocyte mutations.

Increasing melanocyte cAMP levels may be a highly effective melanoma prevention strategy. Therefore, there is great interest in developing novel therapeutics to increase melanocyte cAMP levels in the skin (D'Orazio, Nobuhisa et al. 2006). There are three major approaches for increasing cAMP in melanocytes in the skin. The first mechanism of action is (1) to exploit melanocortin effects on the MC1R using α-MSH or α-MSH analogues (Haskell-Luevano, Shenderovich et al. 1995, Abdel-Malek, Ruwe et al. 2009, Abdel-Malek 2010, Abdel-Malek, Swope et al. 2014). This approach, particularly with topically-applied agents, offers the specificity of melanocyte-directed cAMP stimulation because melanocytes are thought to be the only cells in the skin that express MC1R. The limitation of MC1R-directed therapies, however, is that they would not be expected to be effective in the scenario of loss-of-function MC1R mutations and MC1R-dysfunctional individuals are the most UV-sensitive and prone to melanoma. Thus, melanocortin-based approaches allows more specificity. Other approaches rely on (2) pharmacologic induction of adenylyl cyclase activity (e.g. by forskolin), (3) inhibition of phosphodiesterase activity (e.g. by rolipram) to manipulate melanocyte cAMP levels defective of MC1R status (D'Orazio, Nobuhisa et al. 2006, Passeron, Namiki et al. 2009, Spry, Vanover et al. 2009, Khaled, Levy et al. 2010, Scott, Christian et al. 2012, Amaro-Ortiz, Vanover et al. 2013, Amaro-Ortiz, Yan et al. 2014). These agents, when applied topically, would affect cells of various types rather than being targeted only to melanocytes. Despite their lack of melanocyte specificity, however, these drugs should work even in individuals with homozygous MC1R loss-of-function and provide such persons with UV protection and melanoma prevention. The safe pathway will ensure the safety of melanocyte cAMP stimulation and to design ways of targeting agents to melanocytes in the skin to limit systemic exposure and off-target effects. Topical preparations, for example, offer direct application to the skin, however they may be challenged by ineffective transdermal penetration of the drug or systemic uptake into the

circulation from the skin. Lastly, oral or injectable routing may bypass the need for a transdermal preparation however, systemic effects would be greater. Thus, much work remains to be done to understand the usefulness and potential consequences of manipulating cAMP levels in the skin.

CONCLUSION

Despite a clear understanding of the causative role UV exposure plays in the development of the disease and widespread public health campaigns to limit time spent in the sun or artificial tanning beds, melanoma is a growing and significant public health threat. Loss-of-function polymorphisms of MC1R signaling, which result in reduced melanocyte cAMP levels, strongly correlate with fair complexion, a tendency to burn rather than tan and an increased lifetime melanoma risk. The MC1R/cAMP signaling axis not only leads to the production of UV-protective melanin, but also enhances melanocytic ability to repair UV-damaged DNA. Pharmacologic induction of MC1R pathway provides protection against sunburn. When applied to the skin, forskolin potently increases cutaneous cAMP levels and induces several UV-protective changes in melanocytes. By promoting melanocyte genomic stability, topical forskolin may prove to be a useful UV- and melanoma-preventive strategy.

My work also showed that pharmacologic activation of cAMP/MC1R signaling enhances expression of antioxidant defenses. I studied the mechanistic links between the MC1R/cAMP signaling pathway. I explored the relationships between this pro-survival and pro-differentiation melanocytic signaling pathway and the critical cellular antioxidant enzymes in the skin. I identified, for the first time a possible binding partner of MnSOD after oxidative injury, the chaperone protein HSP70. The mechanism of this interaction might be regulated by PKA, a downstream target of MC1R/cAMP signaling pathway. The use of natural products has significant potential to lessen UV-induced skin damage. This project provides a basic scientific rationale for the development of novel UV-preventive therapeutic agents based on pharmacologic activation of the MC1R/cAMP signaling axis.

APPENDIX A:

GOLDENROD EXTRACT DECREASES THE ANTI-INFLAMMATORY CAPACITY OF FAIR-SKINNED MICE AFTER UV-INDUCED ERYTHEMA IN THE SKIN.

INTRODUCTION

The neural nicotinic acetylcholine receptor alpha 7 (nAChRα7) is a four transmembrane neurotransmitter-gated ion channel (Arredondo, Nguyen et al. 2003). The expression of α7 subunit in the skin promotes keratinocyte adhesion (Arredondo, Nguyen et al. 2002) and attenuation of the pro-inflammatory responses after UV-exposure (Osborne-Hereford, Rogers et al. 2008). The role of this receptor in diseases related to the central nervous system (CNS) has been widely studied specifically in the regulation of the immune response. The CNS can modulate the immune system through the cholinergic anti-inflammatory pathway (Kurzen, Berger et al. 2004). This pathway suppresses the synthesis of cytokines utilizing the neurotransmitter acetylcholine (ACh) as an activator of nAChRα7. ACh is widely distributed and is released in organs of the reticulo-endothelial system such as the gastrointestinal tract, lungs, and kidney among others.

Epidermal keratinocytes produced ACh. The cholinergic pathway is activated by ACh and regulates blood circulation, keratinocytes differentiation (Arredondo, Nguyen et al. 2002), sweat gland formation, and immune reactions. ACh can bind to the nAChRα7 on macrophages and suppresses the release of cytokines and TNF-α. The nAChRα7 can be found in peripheral tissues. The expression nAChRα7 is increased in skin wounds (Fan, Yu et al. 2011). Other studies have focused on how nAChRα7 enhances infiltration of neutrophils in the skin (Gahring, Osborne et al. 2010).

UV radiation is able to induce an inflammatory response in the skin. High doses of UV, can lead to a painful sunburn. When the skin is injured, there is a release of cytokines and chemokine factors to increase permeability and blood flow to the skin. This leads to

swelling and redness of the area affected. If the inflammatory response is excessive, this response can cause harm to cells and surrounding tissues.

Solidago is known as goldenrod and is the state flower of Kentucky. Solidago extract, a natural product, contains many substances including flavonoids. Flavonoids are known for their permeability in vascular capillaries increasing the flow of small molecules or even whole cells to the site of inflammation (Pastore, Potapovich et al. 2009). Oral administration of flavonoids has been used to reduce allergic inflammatory disease (Tanaka 2014). In addition, clinical studies have showed positive results in skin elasticity (Delalle-Lozica 2010).

Solidago extract has been studied as a potential ligand of the nAChRα7 (Lutz, Kulshrestha et al. 2014). Solidago's structure contains an alkaloid methyllycaconitine (MLA) complex that has high affinity for the receptor. It can selectively bind to nAChRα7, leading to its activation modulating the inflammatory response at the site of injury (Lutz, Kulshrestha et al. 2014). The nAChRα7 is found in non-neuronal tissues such as the skin. It has a role in keratinocyte adhesion, cell migration, differentiation and apoptosis (Kurzen, Berger et al. 2004).

Here I studied the role of Solidago extract in skin after UV exposure. Solidago extract is a natural product that can be administered topically. I tested the capacity of solidago extract to reduce UV-induced inflammatory responses in the skin using *K14-SCF* Mc1r-deficient mice. For this study, the mice used were fair-skinned due to decreased MC1R signaling. Albino *extension* mice (Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$) lacking both MC1R signaling and melanin biosynthesis were also used. I tested the capacity of short-term application of crude solidago extract to protect against UV-induced sunburn by measuring the minimal erythematous dose (MED). Moreover, I investigated gene expression in the skin, focusing on cytokines involved in the inflammatory response.

SPECIFIC AIM:

To determine the effect of Solidago against UV-induce inflammation of the skin in fair-skinned mouse model

1a)   To determine the role of solidago after a short-term topical application.

1b)   To determine the MED after solidago treatment.

1c)   To examine the expression of different cytokines and its receptors in the skin.

METHODS AND MATERIALS

Protocols for murine experiments followed the guidelines for ethical conduct in the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Protocol # 00768M2004).

MOUSE COLONY

4 to 12 week old C57BL/6 *extension* non-transgenic and *K14-SCF* transgenic *extension* males and females mice were used. The characteristic phenotype was blonde fur, pink skin, dark footpads and nose tip. The mice showed pheomelanotic fur, pheomelanotic epidermal skin (due to presence of interfollicular epidermal melanocytes).

REAGENTS AND MATERIALS

The following reagents were purchased commercially: Coleus Forskohlii 20% extract (Buckton Scott USA Inc, Princeton NJ); Depilatory cream Nair (Church & Dwight, Princeton , NJ); Xylazine (Anased Injection, Shanandoah, IA); Ketamine (Putney, St. Joseph, MO.); Isothesia Isoflurane (Butler Schein, Dublin, OH); Propyl glycol (Amresco, Solon, OH) and Ethyl Ethanol (Fisher, Waltham, MA.). The following materials were purchased commercially: Chromameter CR-400 and data processor (Konica Minolta, Ramsey, NJ); Electric Shears (Oster, Atlanta, GA); NIST Radiometer/Photometer Model

IL1400A, International Light, Newburyport, MA), and Germicidal Hg Lamb UV-B (Westinghouse, Pittsburgh, PA).

PREPARATION OF SOLIDAGO FOR TOPICAL ADMINISTRATION.

Dr. John Littleton from the College of Pharmacy at the UKY provided the extract for Solidago. The preparation of Solidago was made by solubilizing the extract 10% weight/volume in a standard dermatologic base of 70% ethanol, 30% propylene glycol.

SOLIDAGO (GOLDENROD) TOPICAL TREATMENT

The skin biopsies were obtained from MC1R$^{e/e}$ Tyr$^{+/+}$ C57BL/6 animals unless otherwise stated. The mice were briefly anesthetized using an intraperitoneal injection of a standard mixture of ketamine (Putney, St. Joseph, MO.) and xylazine (Butler Schein, Dublin, OH.) (Typically 0.04 ml per 10g body weight of a mixture of 10 mg/ml ketamine and 1.0 mg/ml xylazine). The dorsal fur was removed using electric shears equipped with a 0.25mm surgical preparatory head (Fisher Scientific) followed by chemical depilation by Nair (Church and Dwight, Princeton, NJ.). After 24 hours, the mice were treated in the dorsal skin with 400uL of 10 % solidago extract; vehicle control animals received 70% ethanol, and 30% propylene glycol alone. Twice daily for 3 days (6 applications) or one day applications were performed for the study. Then, 48 hours after the last topical treatment, the mice were anesthetized with ketamine and xylazine so that UV sensitivity by calculation of "Minimal Erythematous Dose" (MED) could be determined. A UV-occlusive tape was placed on the back of the mice. The mice were placed in a UV source consisting of two Westinghouse F15T8UV-B lamps with a peak output of 313nm and a range of 280 to 370 nm. The UV dose was based on the UV transmission rate and it was measured by a UV Photometer. The UV exposure time was calculated for each desired dose based on the UV output of the source. The mice were monitored for 24 to 48 hours for discreet areas of erythema (redness) or edema (swelling) corresponding to the anatomic sites

exposed to the specific dose of UV irradiation. The skin findings were documented photographically.

MRNA ISOLATION FROM TISSUE SAMPLES

The skin biopsies were taken at desired time-points. Frozen skin tissues were triturated using liquid nitrogen with a mortal and pedestal. The skin was homogenized with Trizol® reagent following manufacturer protocol. The mRNA was clean-up using the RNeasy kit (Qiagen, Valencia, CA.) following the manufacturer protocol. The pure mRNA was quantified, normalized and converted to cDNA using the iScript cDNA kit (Qiagen, Valencia, CA.).

qPCR

The cells were plated in 35mm dishes and treated for cAMP stimulation. Treatments included 10μM forskolin or 0.1% vehicle control and untreated cells as the control samples. The cells were dissociated by treatment with 0.25% trypsin/EDTA at 37°C for 5 min. The cells were harvested, centrifuged and RNA was isolated using the RNAeasy kit (Qiagen, Valencia, CA.). RNA was converted to cDNA using the iScript cDNA kit (Qiagen, Valencia, CA.). The cDNA was used to perform quantitative PCR assay by the CF-96 Biorad qPCR with the Actin β as a reference gene included for each reaction. The levels of expression of genes in stimulated cells were compared with the untreated and control groups.

STATISTICAL ANALYSIS

The data was analyzed between cohorts of replicates samples (n = 3) by one-way ANOVA using the Bonferroni post-test (Graph Pad PRISM software). p values <0.01 were considered statistically significant.

RESULTS

SOLIDAGO DECREASES MED IN MC1R$^{E/E}$ TYR$^{+/+}$ MICE AFTER UV-EXPOSURE.

I studied the effects of topical treatment of solidago (goldenrod) extract on preventing UV-induced skin inflammation using the *K14-SCF* Mc1r$^{e/e}$ Tyr$^{+/+}$ mouse model (Fig 5-1). I tested the role of Solidago in the skin by measuring minimal erythematous dose, MED. Topical administration of Solidago extract had no effect on darkening of the skin (Figure 5-2C, D). After applying solidago twice daily for 3 days (10% Solidago per dose), the average MED for *K14-SCF extension* (Mc1r $^{e/e}$ Tyr $^{+/+}$) mice was significantly higher with 10.0 ± 0.0 kJ/m^2 compared to 4.0 ± 0.0 kJ/m^2 for the vehicle control treated group (Fig. 5-2 C). The average MED in *K14-SCF* albino *extension* (Mc1r $^{e/e}$ Tyr $^{c2j/c2j}$) mice treated with solidago and vehicle were 3.3 ± 1.2 kJ/m^2 and 0.8 ± 0.4 kJ/m^2, respectively (Fig. 5-2 D). There was no significant difference in the erythema of the skin for albino *extension* mice treated with solidago. Here I concluded that Solidago decreased UV-induced inflammation in fair-skinned mice.

ONE TOPICAL TREATMENT OF SOLIDAGO IN MC1R$^{E/E}$ TYR$^{+/+}$ MICE AFTER UV-EXPOSURE DID NOT DECREASE MED.

I performed another experiment with only one application of 10% solidago followed by UV-exposure in *K14-SCF extension* (Mc1r $^{e/e}$ Tyr $^{+/+}$) mice (Figure 5-3). Two groups of mice were pretreated with solidago for 3 or 6 hours before UV exposure. The average MED for the 3 hours group was 2.0 ± 0.0 kJ/m^2 for both solidago and vehicle control. However, for the mice irradiated 6 hours after solidago treatment, the MED was 4.0 ± 0.0 kJ/m^2 compared to 2.0 ± 0.0 kJ/m^2 for vehicle control (Figure 5-3B). Although the MED was decreased, these results were not significant. There was no significant difference in both groups. I concluded that one application of 10% solidago was not enough to promote an anti-inflammatory response.

SOLIDAGO DECREASE THE INFLAMMATORY RESPONSE AT LOWER DOSES OF UVB.

I performed a gene array for the expression of several cytokines and its receptors that are involved in the inflammatory response. Solidago was applied twice daily for 3 days (10% Solidago per dose), to measure MED for *K14-SCF* albino *extension*. Doses of 2kJ/m^2 and 6 kJ/m^2 were used for gene analysis. These doses of UV elicit a low and high inflammatory response in the mouse skin. The MED of K14-SCF albino *extension* (Mc1r $^{e/e}$ Tyr $^{c2j/c2j}$) was measured 48 hours after UV-exposure. The 48 hour time-point was selected to analyze 60 genes that include inflammatory cytokines and its receptor. Out of the genes tested, there were 24 chemokines, 7 chemokine receptors, 7 interleukins, 9 interleukins receptors and others 13 cytokines and its receptors. Of these, there was a decrease in the expression of the majority of pro-inflammatory genes after Solidago treatment compared to vehicle control and a 2 kJ/m^2 dose. There was a decrease in the expression of several cytokines (Ccl1, Cx3cl1, IL15 family among other genes relative to the expression of Actb) (Table 5-1), but there were significant increases in the expression of IL1r1, and Tnfsf13. I noted an increase in the gene expression of several genes after a 6 kJ/m^2 exposure. There was an even higher increase in the expression of IL1r1 and Tnfsf13 were increased by 2 and 6 fold respectively, relative to vehicle. Ccl11 and Cxcl15 had a 2 and 7 fold increase after Solidago treatment, respectively (Table 5-1). Thus, I concluded that topically-applied Solidago decreased the inflammatory response at low doses of UV-exposure.

SOLIDAGO DECREASES Tnf-α SIGNALING IN Mc1R$^{E/E}$ TYR$^{+/+}$ MICE AFTER UV-EXPOSURE.

Previous studies showed the expression of three specific genes as key targets for the inflammatory response (Osborne-Hereford, Rogers et al. 2008). Therefore, I tested the expression of Tnf-α, IL1β and IL6 (Figure 5-4) in *K14-SCF* albino *extension*. The skin biopsies were taken after irradiating the skin with 2 or 6 kJ/m^2 of UV. For a low dose of 2

kJ/m^2, there was a decrease in the gene expression of Tnf-α and IL1β after treatment of 10% solidago compared to vehicle control. But, there was an increase of 4 fold for the gene expression of IL6 after solidago.

Interestingly, for the 6kJ/m^2, there was an increase of 4 fold for IL1β comparing vehicle with solidago treatment. The gene expression of IL6 increased 9 fold after solidago treatment. However, Tnf-α gene expression was decreased 2 fold after solidago treatment. The inhibition of the inflammatory response mediated by solidago may be dependent of the expression of Tnf-α.
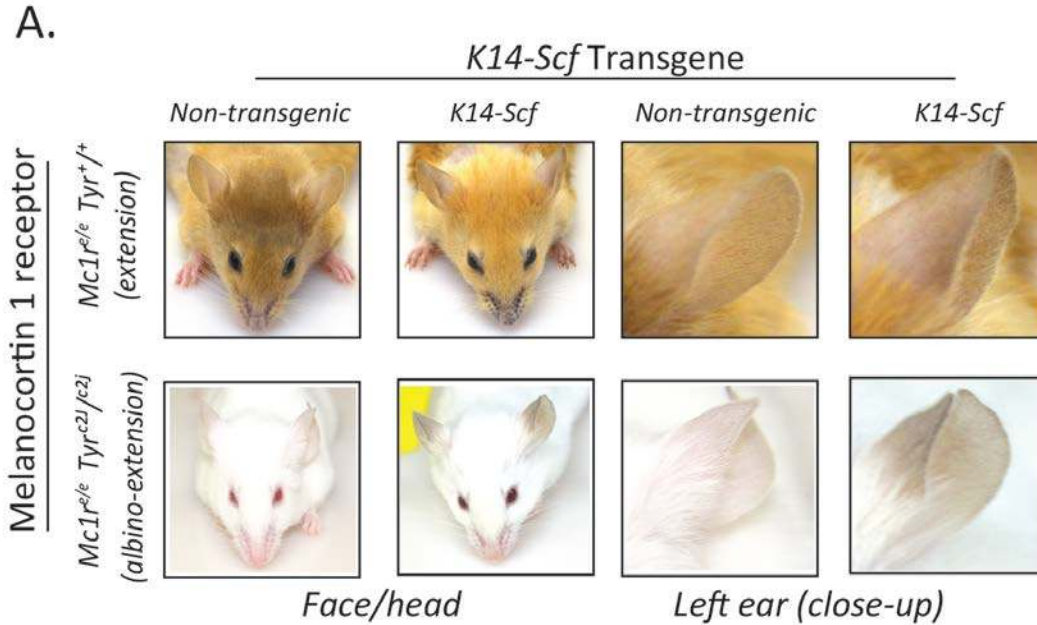
Figure 5-1. Topical treatment of Solidago in fair-skinned *extension* and albino *extension* mice.

(Mc1r$^{e/e}$) mice photographs of C57BL/6 animals were used in this study. Animals were genetically matched except at the melanocortin 1 receptor (Mc1r) and Tyrosinase (Tyr) loci. Note that pigmentation is pheomelanotic (blond) when Tyr is functional but amelanotic (albino) when Tyr is defective, as is the case with the albino *extension* (Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$) mutant. (B) Epidermal pigmentation depends on retention of interfollicular epidermal melanocytes in the skin by the *K14-SCF* transgene, and the skin darkening can easily be seen in the ears.
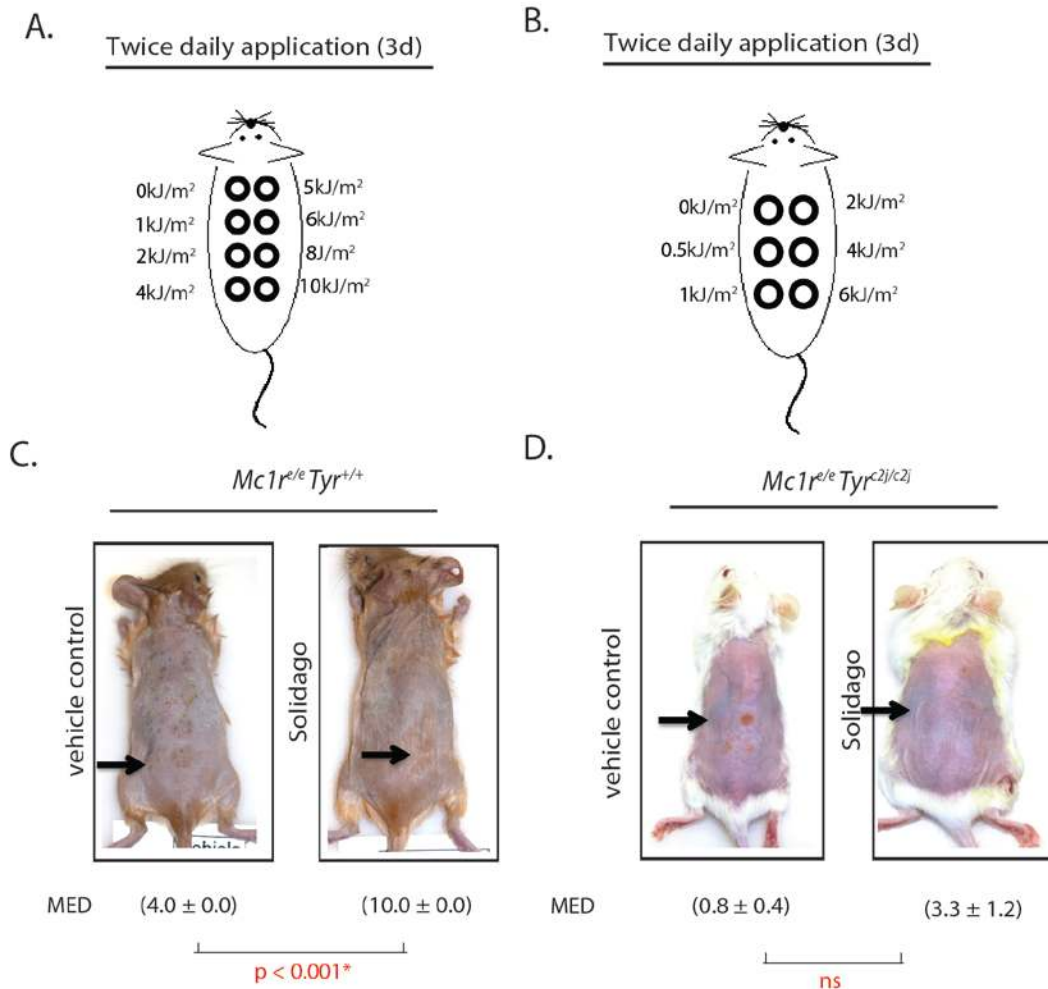
Figure 5-2. Solidago protected against UV-mediated inflammation as determined by minimal erythematous dose (MED) testing.

(A, C) Position of UV occlusive tape and UVB doses of animals treated twice daily for 3 days with 10 % solidago and vehicle control (70% ethanol 30% propyl glycol). Dorsal skin was exposed to various doses of UVB by using UV-occlusive tape with punched-out 1 cm^2 circular apertures, and varying exposure times to yield the appropriate dose. (B, D) Mc1r$^{e/e}$ Tyr$^{+/+}$ and Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$ were irradiated after the completion of the last treatment (6 applications). After irradiation, circles of exposed skin were labeled with a pen in some experiments. MED's, defined by erythema and/or edema in the entire circle of exposed skin to a particular dose, were determined 48 hours after exposure. The MED ± SD results are reported as kJ/m^2 UVB, p ≤ 0.001.
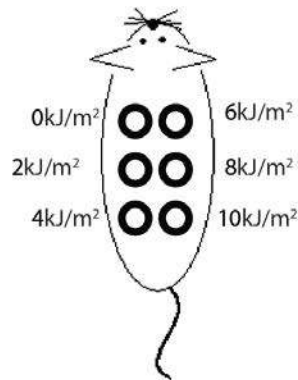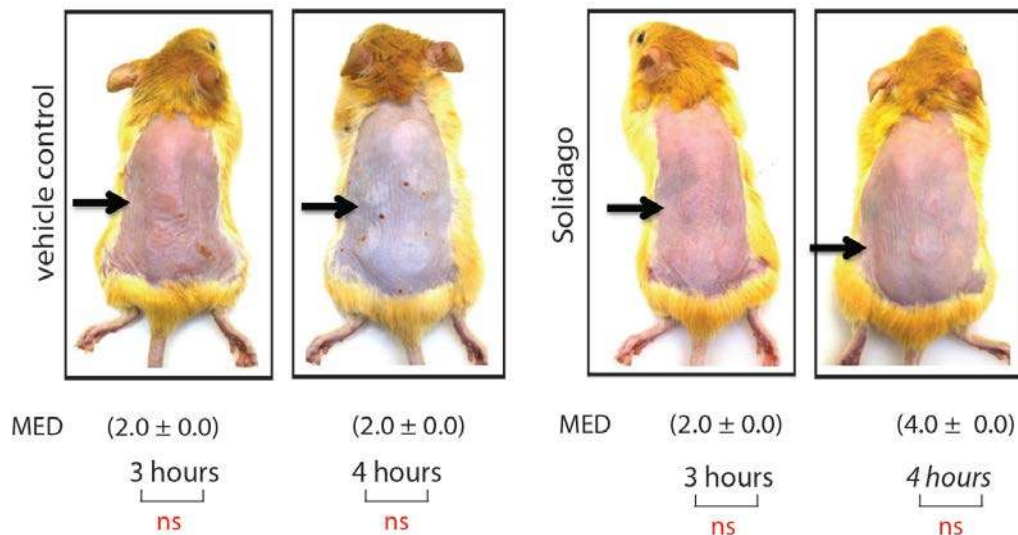
Figure 5-3. Solidago protected against UV-mediated inflammation as determined by minimal erythematous dose (MED) testing.

(A) Position of UV occlusive tape and UVB doses of animals once one day with 10 % solidago and vehicle control (70% ethanol 30% propyl glycol). Dorsal skin was exposed to various doses of UVB by using UV-occlusive tape with punched-out 1 cm^2 circular apertures, and varying exposure times to yield the appropriate dose. (B) $Mc1r^{e/e}$ $Tyr^{+/+}$ mice were treated with solidago 3 and 6 hours prior to UV exposure. After irradiation, circles of exposed skin were labeled with a pen in some experiments. MED's, defined by erythema and/or edema in the entire circle of exposed skin to a particular dose, were determined 48 hours after exposure. The MED ± SD results are reported as kJ/m^2 UVB, * $p \leq 0.001$.

Experiment performed in collaboration with Kaia Hampton.

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Gene Symbol	2kJ/m²	6kJ/m²	Gene Symbol	2kJ/m²	6kJ/m²
Actb	1.000	1.000	Cxcl9	0.501	1.415
Aimp	0.001	1.189	Cxcr2	0.351	1.716
BMP2	0.496	1.057	Cxcr3	0.693	1.918
Ccl1	0.514	1.404	Cxcr5	0.438	0.683
Ccl11	0.570	2.314	Fasl	1.313	1.384
Ccl12	0.184	0.818	Ifng	0.620	1.274
Ccl17	0.889	1.636	IL15	0.132	1.658
Ccl19	0.480	1.424	IL16	0.366	1.050
Ccl2	0.578	1.527	IL17a	0.655	1.149
Ccl20	0.559	1.329	IL17b	0.373	2.850
Ccl22	1.156	1.635	IL17f	0.499	1.548
Ccl24	0.717	2.014	IL1a	0.264	1.985
Ccl3	0.001	0.001	IL1b	0.607	0.859
Ccl4	0.117	1.972	IL1r1	1.814	2.566
Ccl5	0.356	1.669	IL2rg	0.119	1.730
Ccl6	0.732	1.049	IL3	0.082	1.014
Ccl7	0.547	1.741	IL33	0.467	1.614
Ccl8	0.257	0.570	IL4	0.483	1.827
Ccl9	0.486	1.778	IL5	0.412	0.889
Ccr1	0.470	0.952	IL5ra	0.692	2.790
Ccr10	0.511	0.870	IL6ra	0.583	1.444
Ccr6	0.097	0.939	Il6st	0.712	1.266
Ccr8	0.330	0.871	Nampt	0.046	1.109
Cd40lg	0.543	1.958	Osm	0.245	1.292
Csf1	0.511	1.548	Pf4	0.293	0.986
Csf2	0.312	0.973	Spp1	0.404	0.629
Csf3	0.763	2.014	Tnf	0.361	1.140
Cx3cl1	0.633	1.249	Tnfrsf11b	0.411	1.328
Cxcl1	0.199	0.603	Tnfsf10	1.221	2.013
Cxcl15	0.121	7.460	Tnfsf13	6.819	8.349

Table 5-1. Cytokines gene array, the expression of different genes after solidago treatment.

Mc1r$^{e/e}$ Tyr$^{2j/c2j}$ albino *extension* were treated with 10%w/v Solidago and vehicle control (70% ETOH, 30% PG) twice daily for 3 days. Skin biopsies were taken after 48 hours of exposure to 2 or 6 kilojoules of UVB. mRNA was isolated to be analyzed by qPCR using RT^2 Profiler PCR Array for inflammatory cytokines and receptors (Qiagen, Valencia, CA.) with primers specific for mouse mRNA. The gene amplification was normalized to Actin β and the relative amplification levels at both UVB doses were compared to vehicle control.

Experiment performed in collaboration with Kaia Hampton.
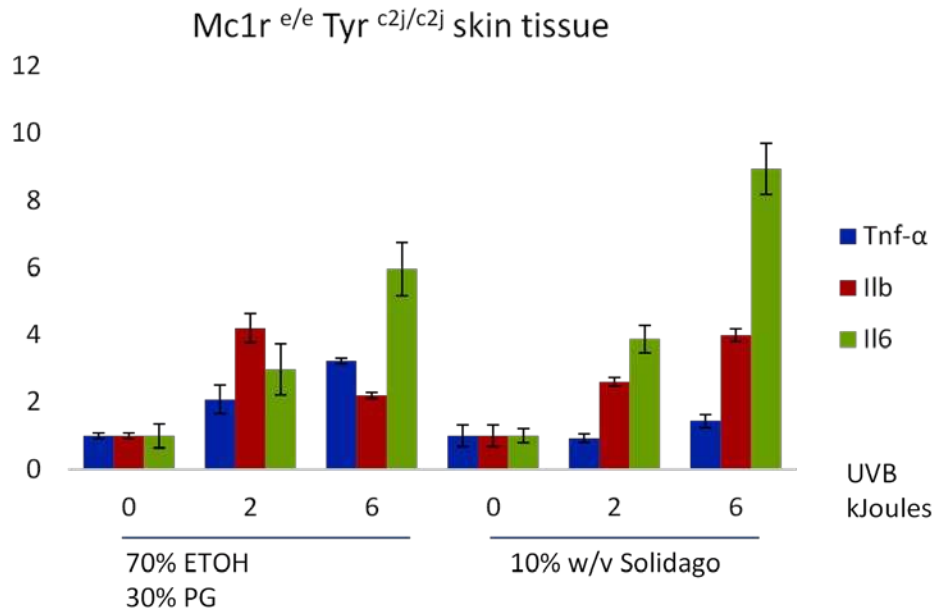
Figure 5-4. Solidago inhibited Tnf-α expression after UV-induced inflammation.

Mc1r[e/e] Tyr[c2j/c2j] albino *extension* were treated with 10%w/v Solidago and vehicle control (70% ETOH, 30% PG) twice daily for 3 days. Skin biopsies were taken after 48 hours of exposure to 2 or 6 kJ/m^2 of UVB. mRNA was isolated to be analyzed by qPCR using RT^2 Profiler PCR Array for inflammatory cytokines and receptors (Qiagen, Valencia, CA.) with primers specific for mouse mRNA. The gene amplification was normalized to Actin β (Actb) and the relative amplification levels at both UVB doses were compared to vehicle control.

Experiment performed in collaboration with Kaia Hampton.

DISCUSSION

The anti-inflammatory response of solidago is mediated by its binding to nAChRα7 This receptor has been found to be expressed in the skin (Zia, Ndoye et al. 2000, Arredondo, Nguyen et al. 2002, Bencherif, Lippiello et al. 2011, Fan, Yu et al. 2011). I hypothesized that the solidago extract would protect against UV-inflammation in *K14-SCF* Mc1r$^{e/e}$ Tyr$^{+/+}$ and Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$ mice. This is the first study using a topical formulation of solidago. I confirmed my hypothesis in C57BL/6 *extension* (Mc1r$^{e/e}$ Tyr$^{+/+}$) mice. I was not able to observe darkening of the skin after 6 applications of the solidago extract on the back of the mice in contrast to forskolin, which induced darkening of the skin after two topical applications. This was not a surprise since the effects of solidago are known to affect inflammatory response rather than pigmentation. However, there was a protective effect against erythema with a 4.0 kJ/m^2 compared to 10.0 kJ/m^2 in Mc1r$^{e/e}$ Tyr$^{+/+}$ fair-skinned mice. I tested the drug in Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$ albino *extension* mice but the increase in MED was not significant. Perhaps, the presence of pheomelanin may have a protective effect against UV-induced inflammation in the skin. To address these differences, further experiments are required with more mice or a higher percentage of solidago extract in the skin. The UV exposure of the Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$ albino *extension* was lower (0.5 to 6.0 kJ/m^2) from Mc1r$^{e/e}$ Tyr$^{+/+}$ *extension* (1.0 to 10 kJ/m^2) because amelanotic albino show more UV sensitivity. I was not able to test higher doses of UV, for the reason that without the darkening of the skin, higher doses of UV would harm the mice and cause excessive sunburn.

The use of solidago in both *extension* and albino *extension* mice, promoted decreases in the inflammatory response. The solidago extract has shown no physiological effect after treatment in mice (Bucciarelli, Minetti et al. 2010). Application of solidago for only one day show no significant effect protecting against UV-inflammation of the skin.

Other studies using chronic treatment (more than 3 days) of topical solidago are needed to study its effect inhibiting the inflammatory response.

Based on the results above, the mechanism of solidago-induced protection is melanin-independent. Skin samples taken from Mc1r$^{e/e}$ Tyr$^{c2j/c2j}$ albino *extension* mice were tested for the expression of several genes involved in the inflammatory response. The gene expression of the majority of cytokines and receptors was decreased at low UV but not at 6 kJ/m^2 suggesting a protective threshold. However, the expression of IL1r1 and Tnfsf13 was increased in both doses. These cytokines have been used as a target to test inhibition of the inflammatory response (Li, Pappas et al. 2013, Garcia-Perez, Allaeys et al. 2014, Tsai, Tung et al. 2015). The use of flavonoids such as solidago might yield protective effects after UV exposure perhaps more effective at lower doses of UV.

The inflammatory response consists of many cytokines receptors. An example of pro-inflammatory cytokines include IL23, IL17, IL6, IL8, IL10, and Tnf-α among others. IL6 is expressed in severe skin sunburn (Norval 2001, Norval 2011). IL6 promotes the release of Tnf-α, IL12, IL1 and other cytokines promoting inflammation. IL6 showed a high expression when tested in the skin treated with 6 kJ/m^2 of UV radiation on albino *extension* mice confirming its role in skin sunburn. Tnf-α is a pro-inflammatory cytokine and solidago decreased it at both low and high doses of UV. On the other hand, there are anti-inflammatory cytokines like IL13, IL11, and IL4. IL4 and IL13 known to promote tissue repair together with the production of specific antibodies (Voehringer 2013), therefore some beneficial effects of inflammation may be lost by Solidago treatment.

I also noted decreases in the expression of Tnf-α and IL1β after solidago treatment at lower doses of UVB. The decrease remained after increasing the dose of UVB to 6 kJ/m^2 for Tnf-α. However, IL1β and IL6 showed an increase in the expression after an exposure of 6 kJ/m^2. This dose of UVB showed an increase in skin redness of the mice.

The protective effect of solidago at higher doses of UVB might be linked to the regulation of Tnf-α. Remarkably, Tnf-α is known to regulate dendritic and macrophages density in the immune response (Marble, Gordon et al. 2007) and IL6 can inhibit endotoxin-induced Tnf-α signaling (Starkie, Ostrowski et al. 2003). Additional studies are required to determine if solidago can directly regulate IL6 as an anti-inflammatory cytokine.

The use of solidago for the prevention of inflammation after UVB exposure would potentially be of benefit to individuals with UV sensitivity. It is important to consider that the solidago extract contains many other components that might have a role in the skin response. Therefore, further studies are needed to understand the absorption of this drug in the skin and to clarify which components are inflammation regulators. Better absorption might allow more significant results at higher doses of UV. It would be ideal to observe an anti-inflammatory effect using less than 6 application of solidago (in a matter of hours).

I hypothesized that solidago acted as an agonist to the nAChrα7 to decrease cytokine production. Although nAChRα7 has been implicated in wound healing (Fan, Yu et al. 2011) and skin infection (Gahring, Osborne et al. 2010), its pharmacologic activation in the absence ACh or parasympathetic innervation is a novel mechanism. The nAChRα7 being expressed in non-neuronal locations regulates early local inflammatory responses in the skin. This suggests that the effect of this receptor might be beneficial even without UV-exposure. In support of our hypothesis, it is clear that several pro-inflammatory cytokines were down-regulated at the gene level after topical application of solidago activates nAChRα7 response and inhibits the inflammatory response.

APPENDIX B:

PERMISSION LETTER TO RE-USE FIGURES

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Hi Alexandra,

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LIST OF ABBREVIATIONS

6-4 PP	6-4 photoproduct
8-oxoG	8-oxoguanine
A	adenine
ACTB	Actin, beta
ADIPOQ	adiponectin, C1Q and collagen domain containing
ADP	Adenosine diphosphate
AGTR1	angiotensin II receptor, type 1
AIMP1	Aimp1 Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1
AKT1	v-akt murine thymoma viral oncogene homolog 1
APEX1	nuclease (multifunctional DNA repair enzyme) 1
APOA1	apolipoprotein A-I
APP	amyloid beta (A4) precursor protein
ARE	antioxidant respose element
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
ATP	Adenosine triphosphate
B2M	B2m Beta-2 microglobulin
BCL2	B-cell CLL/lymphoma 2
BMP2	Bmp2 Bone morphogenetic protein 2
C	cytosine
cAMP	3'-5'-cyclic adenosine monophosphate
CASP3	caspase 3, apoptosis-related cysteine peptidase
CAT	catalase
CCL1	Chemokine (C-C motif) ligand 1
CCL11	Chemokine (C-C motif) ligand 11
CCL12	Chemokine (C-C motif) ligand 12
CCL17	Chemokine (C-C motif) ligand 17
CCL19	Chemokine (C-C motif) ligand 19
CCL2	Chemokine (C-C motif) ligand 2
CCL20	Chemokine (C-C motif) ligand 20
CCL22	Chemokine (C-C motif) ligand 22
CCL24	Chemokine (C-C motif) ligand 24
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CCL6	Chemokine (C-C motif) ligand 6
CCL7	Chemokine (C-C motif) ligand 7
CCL8	Chemokine (C-C motif) ligand 8
CCL9	Chemokine (C-C motif) ligand 9
CCR1	Chemokine (C-C motif) receptor 1
CCR10	Chemokine (C-C motif) receptor 10
CCR2	Chemokine (C-C motif) receptor 2
CCR3	Chemokine (C-C motif) receptor 3
CCR4	Chemokine (C-C motif) receptor 4
CCR5	Chemokine (C-C motif) receptor 5

CCR6	Chemokine (C-C motif) receptor 6
CCR8	Chemokine (C-C motif) receptor 8
CD36	molecule (thrombospondin receptor)
CD40LG	CD40 ligand
CLU	clusterin
CPD	cyclobutane pyrimidine dimer
CREB	cAMP response element binding
CSF1	Colony stimulating factor 1 (macrophage)
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
CSF3	Colony stimulating factor 3 (granulocyte)
Cu/ZnSOD	copper/zinc superoxide dismutase
CX3CL1	Chemokine (C-X3-C motif) ligand 1
CXC12	Chemokine (C-X-C motif) ligand 12
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL11	Chemokine (C-X-C motif) ligand 11
CXCL13	Chemokine (C-X-C motif) ligand 13
CXCL15	Chemokine (C-X-C motif) ligand 15
CXCL5	Chemokine (C-X-C motif) ligand 5
CXCL9	Chemokine (C-X-C motif) ligand 9
CXCR2	Chemokine (C-X-C motif) receptor 2
CXCR3	Chemokine (C-X-C motif) receptor 3
CXCR5	Chemokine (C-X-C motif) receptor 5
CYBA	cytochrome b-245, alpha polypeptide
CYCS	cytochrome c, somatic
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1
DCT	dopachrome tautomerase
DDIT3	DNA-damage-inducible transcript 3
DHCR24	24-dehydrocholesterol reductase
DHICA	5,6-dihydroxyindole-carboxylic acid
ECSOD	extracellular superoxide dismutase
EGFR	epidermal growth factor receptor
F3	coagulation factor III (thromboplastin, tissue factor)
FASL	Fas ligand (TNF superfamily, member 6)
FBS	Fetal Bovine Serum
FOXO1	forkhead box O1
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCLC	glutamate-cysteine ligase, catalytic subunit
GCLM	glutamate-cysteine ligase, modifier subunit
GLRX	glutaredoxin (thioltransferase)
GLRX2	glutaredoxin 2
GPCR	g-protein coupled receptor
GPX1	glutathione peroxidase 1
GPX3	glutathione peroxidase 3 (plasma)
GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)
GSH	glutathione
GSR	glutathione reductase
GSTP1	glutathione S-transferase pi 1
GUSB	Glucuronidase, beta

H$_2$O$_2$	Hydrogen Peroxide
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HMOX1	heme oxygenase (decycling) 1
HP	haptoglobin
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSP90A1	Heat shock protein 90 alpha (cytosolic), class B member 1
HSPD1	heat shock 60kDa protein 1 (chaperonin)
IFNG	Interferon gamma
IL10RA	Interleukin 10 receptor, alpha
IL10RB	Interleukin 10 receptor, beta
IL11	Interleukin 11
IL13	Interleukin 13
IL15	Interleukin 15
IL16	Interleukin 16
IL17A	Interleukin 17A
IL17B	Interleukin 17B
IL17F	Interleukin 17F
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1 beta
IL1B	Interleukin 1, beta
IL1R1	Interleukin 1 receptor, type I
IL1RN	Interleukin 1 receptor antagonist
IL21	Interleukin 21
IL27	Interleukin 27
IL2RB	Interleukin 2 receptor, beta chain
IL2RG	Interleukin 2 receptor, gamma chain
IL3	Interleukin 3
IL33	Interleukin 33
IL4	Interleukin 4
IL5	Interleukin 5
IL5RA	Interleukin 5 receptor, alpha
IL6	Interleukin 6 (interferon, beta 2)
IL6RA	Interleukin 6 receptor, alpha
IL6ST	Interleukin 6 signal transducer
IL7	Interleukin 7
INS	insulin
IP	Intraperitoneal
JAK2	Janus kinase 2
JUN	proto-oncogene
kDA	Kilodalton
LCN2	lipocalin 2
LDHA	lactate dehydrogenase A
LTA	Lymphotoxin A
LTB	Lymphotoxin B
MAP3K5	mitogen-activated protein kinase kinase kinase 5
MAPK1	mitogen-activated protein kinase 1
MAPK8	mitogen-activated protein kinase 8
MAPK9	mitogen-activated protein kinase 9
MC1R	melanocortin 1 receptor
α-MSH	alpha melanocyte stimulating hormone

MDM2	p53 binding protein homolog (mouse)
MED	minimal erythemal dose
MGDC	Mouse Genomic DNA Contamination
MIF	Macrophage migration inhibitory factor
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
Mitf	micropthalmia transcription factor
MnSOD	manganese superoxide dismutase
MPO	myeloperoxidase
MSRA	methionine sulfoxide reductase A
mtDNA	mitochondrial DNA
NAMPT	Nicotinamide phosphoribosyltransferase
NFE2L2	nuclear factor (erythroid-derived 2)-like 2
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NMSC	non-melanoma skin cancer
NOS1	nitric oxide synthase 1 (neuronal)
NOX1	NADPH oxidase 1
NQO1	NAD(P)H dehydrogenase, quinone 1
Nrf2	Nuclear factor-like 2
OGG1	8-oxoguanine DNA glycosylase
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
OSM	Oncostatin M
PARK7	Parkinson disease (autosomal recessive, early onset) 7
PBS	Phosphate Buffer Saline
PF4	Platelet factor 4
PKA	protein kinase A
POMC	proopiomelanocortin
PON2	paraoxonase 2
PRDX1	peroxiredoxin 1
PRDX2	peroxiredoxin 2
PRDX3	peroxiredoxin 3
PRDX4	peroxiredoxin 4
PRDX5	peroxiredoxin 5
PRDX6	peroxiredoxin 6
PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit
PRKCA	protein kinase C, alpha
PRKCB	protein kinase C, beta
PRKCD	protein kinase C, delta
PRKCZ	protein kinase C, zeta
PRODH	proline dehydrogenase (oxidase) 1
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
RHC	red hair color phenotype
RNS	reactive nitrogen species
ROS	reactive oxygen species
ROS1	c-ros oncogene 1, receptor tyrosine kinase
SCF	stem cell factor

SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SHC1	SHC (Src homology 2 domain containing) transforming protein 1
SIRT1	sirtuin 1
SNCA	synuclein, alpha (non A4 component of amyloid precursor)
SOD	superoxide dismutase
SOD1	superoxide dismutase 1, soluble
SOD2	superoxide dismutase 2, mitochondrial
SOD3	superoxide dismutase 3, extracellular
SPP1	Spp1 Secreted phosphoprotein 1
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAT1	signal transducer and activator of transcription 1, 91kDa
TBS-T	tris buffered saline – tween 20
TLR4	toll-like receptor 4
TNF	Tumor necrosis factor
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11
TNFSF13	Tumor necrosis factor (ligand) superfamily, member 13
TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b
TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4
TP53	tumor protein p53
TXNIP	thioredoxin interacting protein
TXNRD1	thioredoxin reductase 1
Tyr	tyrosinase
Trp1	tyrosine related protein 1
Trp2	tyrosine related protein 2
UCP1	uncoupling protein 1
UCP2	uncoupling protein 2 (mitochondrial, proton carrier)
UV	ultraviolet
UVA	ultraviolet A
UVB	ultraviolet B
UVC	ultraviolet C
VDAC	voltage-dependent anion channel
VEGFA	Vascular endothelial growth factor A
XO	xanthine oxidase

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Alexandra Amaro-Ortiz

Education

08/10-present	Ph.D., Toxicology, University of Kentucky, Lexington, KY
08/09-06/10	Post B.S., Pharmaceutical Science, University of Puerto Rico, Medical Science Campus. San Juan, PR
08/05-05/09	B.S., Chemistry, University of Puerto Rico Rio Piedras Campus, San Juan, PR

Professional Experiences

08/10-present	Graduate Research Assistant, University of Kentucky, Lexington KY
08/09-07/10	Graduate Research Assistant, University of Puerto Rico, San Juan, PR
06/08-08/08	Undergraduate Research Assistant, University of Arizona, Tucson, AZ
08/07-05/08	Undergraduate Research Assistant, University of Puerto Rico, San Juan, PR
06/07-08/07	Undergraduate Research Assistant, University of Kentucky, Lexington KY

Laboratory Rotation trainees

10/12-4/14	Betty Yan, Medical student, Professional Student Mentored Research Fellowship, University of Kentucky (18 months)
10/12-4/14	Claci Ayers, Undergraduate Student, Honors Program, Univ. KY. (18 months)
10/13-12/13	Kaia Hampton, IBS Graduate Student, University of Kentucky (8 weeks)
6/12-8/12	Brenda Patel, Undergraduate Student from Georgetown College. Summer research for undergraduate students as part of the HHMI-funded Georgetown College Program to Accelerate Learning in the Sciences (GCPALS). (10 weeks)
1/12-3/12	Jared Vasquez, IBS Graduate Student, University of Kentucky (8 weeks)
10/11-12/11	Karine Oden, IBS Graduate Student, University of Kentucky (8 weeks)

Awards and/or Scholarships

08/10-03/12	Lyman T. Johnson Fellowship, $7,500 per year plus ½ tuition. Remaining support provided by Graduate Center for Toxicology departmental funds.
06/08-05/09	SMART Scholarship for Undergraduate Studies 2008-2009 -For undergraduate students to complete the B.S. degree.
06/05-05/09	Robert C. Bird Scholarship for Undergraduate Studies 2005-2009 -For undergraduate students to complete the B.S. degree.
06/05-07/06	The Ronald McDonald Foundation Scholarship- For first year undergraduate students enroll in a college degree.
12/06	Travel Award to Puebla, Mexico – As a member of P.E.C.E.S., Inc. To encourage primary and secondary school students to pursue a career.

Scientific publications

Original Article

Amaro-Ortiz, A., Vanover, J., Scott, T.L. and D'Orazio, J. 2013. *Pharmacologic induction of epidermal melanin and protection against sunburn in a humanized mouse model.* J Vis Exp. 2013(79).

Book Chapter

A. Amaro-Ortiz, M. Boulanger, and J. D'Orazio. 2015. *"UV Protection of the Skin by Topically-applied Forskolin",* in Forskolin: Sources, Mechanisms of Action and Health Effects, Nova Science Publishers, Inc., Hauppauge, NY.

Jarrett, S., J. Tucker, **A. Amaro-Ortiz** and J. D'Orazio. 2013. "*Melanocortin-1 receptor: a critical regulator of the UV DNA damage repair response*", in Skin Pigmentation: Genetics, Geographic Variation and Disorders, Nova Science Publishers, Inc., Hauppauge, NY.

Review Article

Amaro-Ortiz, A, Yan B, & D'Orazio JA. Ultraviolet Radiation, Aging and the Skin: Prevention of Damage by Topical cAMP Manipulation. *Molecules*. 2014; 19(5):6202-6219.

D'Orazio, J., Jarrett, S., **Amaro-Ortiz, A.** and T. Scott. 2013. "UV Radiation and the skin", in a Special Issue: Radiation Toxicity in Cells, *Int. J. Mol. Sci.* 2013, *14*(6), 12222-12248.

Abstracts and Lectures

8/14   Amaro-Ortiz, A., Ayer, C., Yan, B. and J.A. D'Orazio. Pharmacologic induction of the melanocortin 1 receptor (MC1R) pathway provides protection against sunburn and enhances expression of MnSOD in the skin.
        2014 Natural Product Consortium Symposium, Lexington, KY

5/14   Amaro-Ortiz, A., Ayer, C., Yan, B. and J.A. D'Orazio. The melanocortin 1 receptor (MC1R) pathway enhances expression of MnSOD and protects against ROS-induced oxidative stress in human melanocytes.
        2014 Markey Cancer Research Day, University of Kentucky, Lexington, KY.

5/14   Amaro-Ortiz, A., Ayer, C., Yan, B. and J.A. D'Orazio. The melanocortin 1 receptor (MC1R) pathway enhances expression of MnSOD and protects against ROS-induced oxidative stress in human melanocytes.
        16th Annual Midwest DNA Repair Symposium, Detroit, MI.

11/13   Amaro-Ortiz, A. Melanocortin 1 receptor (Mc1r) signaling mediates protection against skin cancer.

3MT competition. University of Kentucky, Lexington, KY

10/13   Amaro-Ortiz, A., S.G. Jarrett, C.W. Allen and J.A. D'Orazio. Melanocortin 1 receptor (Mc1r) signaling mediates up-regulation of anti-oxidant enzymes. *2nd Annual Appalachian Regional Cell Conference, Charleston, W.V.*

8/13   Amaro-Ortiz, Alexandra. Lecture: The role of MC1R/cAMP signaling pathway in antioxidant defenses. *2013 Summer Research Program Trainees.*

5/13   Amaro-Ortiz, A., S.G. Jarrett, C.W. Allen and J.A. D'Orazio. Melanocortin 1 receptor (Mc1r) signaling mediates up-regulation of anti-oxidant enzymes and protects against UV-induced mitochondrial damage. *15th Annual Midwest DNA Repair Symposium, Lexington, KY. (Poster Award, Second Place)*

5/13   Amaro-Ortiz, A., S.G. Jarrett, C.W. Allen and J.A. D'Orazio. Melanocortin 1 receptor (Mc1r) signaling upregulates anti-oxidant enzymes and protects against mitochondrial damage. *2013 Markey Cancer Center Research Day, Lexington, KY.*

5/12   Amaro-Ortiz, A., P. Murapa and JA D'Orazio. "Melanocytic anti-oxidant enzymes are regulated by melanocortin 1 receptor (Mc1r) signaling". *14th Annual Midwest DNA Repair Symposium, Cincinnati, OH.*

5/12   Amaro-Ortiz, A., P. Murapa and JA D'Orazio. "Melanocytic anti-oxidant enzymes are regulated by melanocortin 1 receptor (Mc1r) signaling", *2012 Markey Cancer Center Research Day, Lexington, KY.*

03/09   Puerto Rico Interdisciplinary Scientist Meeting (PRISM) and 5rd Junior Technical Meeting. *San Juan, PR*

11/08   Annual Biomedical Research Conference for Minority Students. *Orlando, FL*

03/08   Puerto Rico Interdisciplinary Scientist Meeting (PRISM) and 4rd Junior Technical Meeting. *Arecibo, PR*