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Modulation of GRKs and beta-arrestins expression by UVB in the keratinocyte cell line HaCaT and the epidermis

Diploma thesis

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V Hradci Králové 2012

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1 ABBREVIATION LIST

AEBSF	4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride
APS	Ammonium persulfate
α-MSH	Alpha melanocortin stimulation hormone
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
DMEM	Dulbecco´s modified Eagle's medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ECL	Extracellular loop
FBS	Fetal bovine serum
GDP	Guanosine diphosphate
G protein	GTP-binding protein
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GTP	Guanosine triphosphate
HEPES acid	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
ICL	Intracellular loop
IL-1	Interleukin-1
MC1R	Melanocortin 1 receptor
NF-κB	Nuclear factor kappa B
NP-40	Nonyl phenoxypolyethoxylethanol
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBP	TATA-binding protein
TBS	Tris-buffered saline
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
TNF-α	Tumor necrosis factor-α
TRIS	Tris-hydroxymethyl-aminomethane
TWEEN 20	Polyethylen sorbitan monolaurate
UV	Ultraviolet

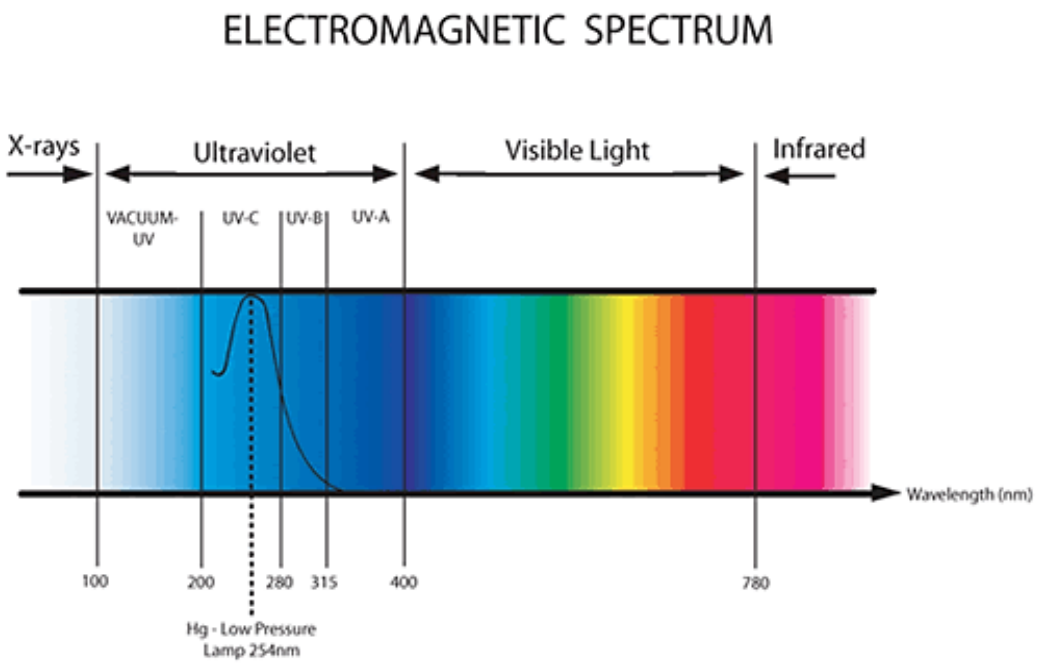
2 INTRODUCTION

2.1 Electromagnetic spectrum

Sunlight consists of a continuous spectrum of electromagnetic radiation which is divided into three main components of wavelengths - ultraviolet (UV) (45 %), visible (5 %) and infrared (50 %). The UV light area appears between 100–400 nm (see **Figure 1**)**.**
In compliance with the International Commission on Illumination, UV radiation is divided into three groups depending on the wavelength *(Svobodova et al., 2006):*

- long wave UVA (320–400 nm)
- medium wave UVB (280–320 nm)
- short wave UVC (200–280 nm)

Figure 1*: Electromagnetic spectrum*



The Electromagnetic spectrum is composed of X-rays (wavelength 100 nm), ultraviolet spectrum (100 - 400 nm), visible light (400-780 nm) and infrared (above 780 nm). Ultraviolet radiation is divided into Vacuum UV (100-200 nm), UVC (200-280 nm), UVB (280 – 320 nm) and UVA (320 – 400 nm).
(adapted from: http://www.aquafineuv.com/UVTechnology/UVScience.aspx)

UV radiation is efficiently absorbed by the ozone layer up to about 310 nm, thus it absorbs all UVC and most UVB (95 %). Because of the substantial

damage to the protective ozone layer, a greater amount of UVB radiation reaches the ground.

However, UVA is not absorbed at all. UVA is contained in more than 95 % of solar radiation that reaches us. In comparison with UVA and UVB, UVB is long wave radiation which penetrates deeply into the epidermis and dermis of the skin. UVB is also about 1000 times more effective in the production of a direct sunbathing effect, which is made by the darkening of the melanin in the epidermis *(Svobodova et al., 2006).*

However, UV offers health benefits too - sunlight-mediated synthesis of vitamin D, vitamin D_3 to be precise, which is modified by UV radiation from a provitamin to the effective form *(Moon et al., 2005).*

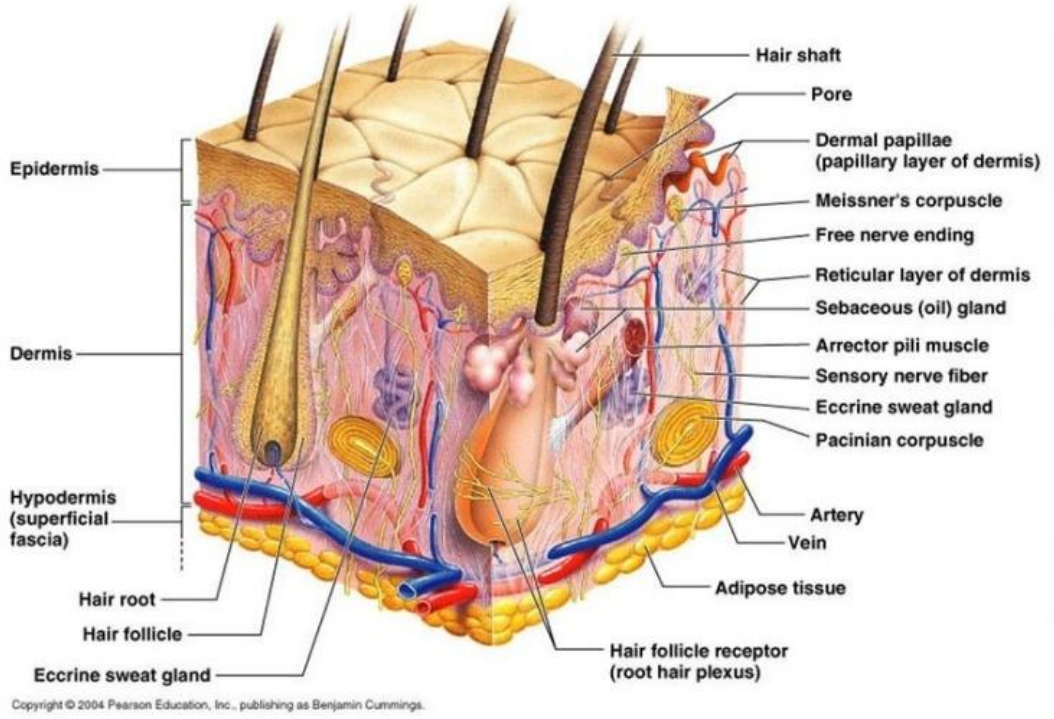
UV light also has strong genotoxic effects such as DNA damage or the induction of mutations and, in the worst case, causes the growth of tumours. The sun is the main natural source of UV and solar UV is known to be one of the main causes of human skin cancers. It has been proved that UVB has a strong carcinogenic effect on the skin and it has also been suggested that UVA is partly involved in carcinogenesis of the skin *(Ikehata and Ono, 2011).*

2.2 Skin

The skin is the largest organ of human body. The skin represents the first line of protection for the body from harmful external effects. Besides this, the skin provides a protective shield against UV. The skin also plays a part in ensuring the stability of the environment inside the body.

As seen in **Figure 2,** the skin is made up of three main layers: the epidermis, dermis and hypodermis - subcutaneous fat layer. Each layer carries out a specific function.

Figure 2: ***Model of Skin***



Anatomical structure of skin which consists of three main layers- epidermis, dermis and hypodermis.
(adapted from: http://www.osovo.com/diagram/skindiagram.htm)

2.2.1 Epidermis

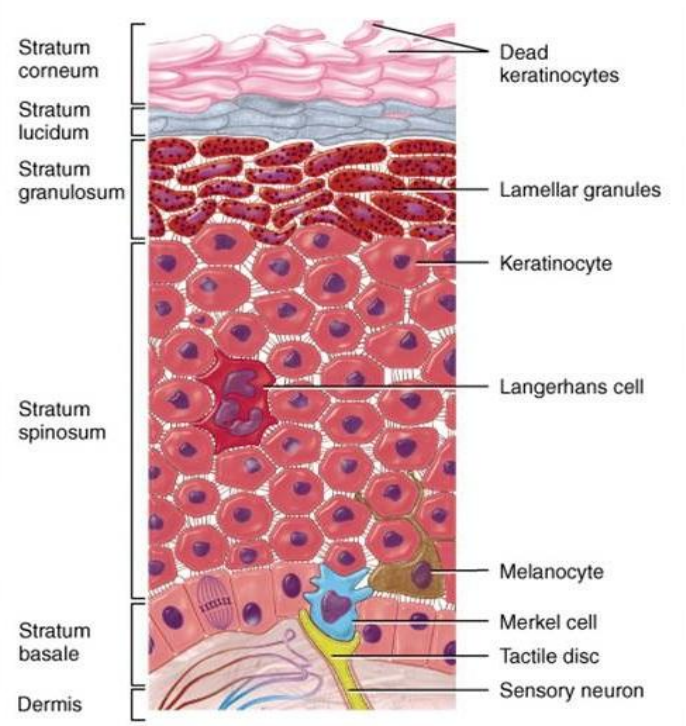
The epidermis contains no blood vessels and it is completely dependant on the underlying dermis for nutrient delivery and waste disposal via diffusion through the dermoepidermal junction. The epidermis is created of multi-layer, squamous, keratinize epithelium. There are five distinguishable layers – stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (see **Figure 3**). The stratum corneum contains primarily keratinocytes that are in progressive stages of differentiation from deeper to more superficial layers.

In the stratum basale there are stem cells which enable the regeneration of the skin. Stem cells are capable of life-long reproduction. One daughter cell retains the original properties of the stem cell and the other cells are subjected to repeated mitotic dividing.

As mentioned above, the keratinocytes move from this deep layer to the more surficial layer. During this process, called keratinization, the keratinocytes lose the ability to reproduce and they are transformed from highly cylindrical keratinocytes to flat and nuclear-free cells. Whole this cycle takes 3-4 weeks.

The cells of the stratum corneum are the largest and the most abundant of the epidermis.

Figure 3: ***Model of epidermis***



The anatomical structure of epidermis consists of the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale that is in contact with the middle layer of skin – dermis.
*(*adapted from:
http://www.imperial.edu/~thomas.morrell/cha_5_tortora_integument.htm)

The melanocytes are situated in the basal layer of the epidermis. These cells are derived from neural crests cells. Their main function is the production of a dark pigment known as melanin. Melanin is accumulated in specific organelles, termed melanosomes, which are incorporated into dendrites anchoring the melanosome to the surrounding keratinocytes.

Melanocytes are sensitive to the UV radiation that causes chemical changes of the pigment. This radiant energy from the sun is absorbed by melanocytes. This process also provides the skin with protection from the harmful effects of UV radiation.

Eventually, the melanosomes are transferred via phagocytosis to the nearby keratinocytes. There, the melanosomes remain in the form of granules. If an accumulation of melanocytes occurs (naevi pigmentosi) during migration of melanocytes to the epidermis, it may be the origin of a malignant tumour -

melanoma can be presented by this particular type of this accumulation. Melanocytes are found in the basal layer of the epidermis as well as in hair follicles and the retina. The production of melanin is stimulated by factors such as, for example, exposure to sunlight, melanocyte-stimulating hormone, adrenocorticotropic hormone, estrogens and progesterones. It has been observed that the number of melanocytes in the skin of an individual decreases with age.

Apart from keratinocytes and melanocytes, the epidermis consists of Langerhans and Merkel cells too. The Langerhans cells originate from the bone marrow and are found in the basal, spinous and granular layers of the epidermis. They are dendritic, antigen-presenting cells that are transported to the dermis via blood. The Langerhans cells are capable of ingesting foreign antigens, processing them into small peptide fragments and binding them with lymphocytes for activation of the immune system. After contact with the antigen they travel to the dermis and back via lymph to the regional lymph node. Contact hypersensitivity and atopic eczema are examples of the activation of immune system.

Like melanocytes, Merkel cells are derived from neural crest cells and situated in the basal layer where they are in the contact with nerve fibre. Merkel cells are conveyed by the process of migration. These cells are mainly concentrated in sinus hair follicles. The Merkel cells function like uncovered receptors, especially for the perception of light touch.

2.2.2 Dermis

The dermis is the middle layer of the skin. Thanks to the dermal papillae, the line between the epidermis and dermis is wavy. The dermis is composed of two parts. The first is the cellular stratum papillare situated in the surface and the second is the deeper located stratum reticular containing elastic and collagen fibres. This fibre is made of strands that are interwoven in a dense net. Due to this collagen structure, skin becomes firm and elastic. Besides blood vessels, lymph vessels and sweat glands, smooth muscles occur in the dermis too. These muscles serve as hair erectors.

Also situated in the dermis are free nerve endings that act as pain receptors. There are also specific Pacinian and Meissner´s corpuscles, which have the function of touch receptors.

2.2.3 Hypodermis

The hypodermis, also known as the subcutaneous layer, is the deepest layer of skin. The hypodermis consists of a collagen network and fat cells that help to conserve the body's heat and protect the body from injury *(web interval*[a,b,c,d]*)*.

2.3 Human keratinocyte cell lines - HaCaT

In recent years, the skin has been shown to contain a broad spectrum of enzymes capable of metabolizing a wide range of topically applied drugs and endogenous substrates. A major part of this metabolic activity is located within the epidermis.

For this reason, there is growing interest in methods for studying human skin metabolism. Since it is difficult to differentiate skin from systemic metabolism under in vivo conditions, there is need for suitable in vitro models. The spontaneously immortalized human skin cell line HaCaT represents a readily available in vitro model and has been already used as a model for skin toxicity studies *(Altenburger and Kissel, 1999).*

The HaCaT is the first permanent epithelial cell line from adult human skin that exhibits normal differentiation and provides a promising tool for studying the regulation of keratinisation in human cell. In contrast to mouse epidermal cells, human skin keratinocytes are rather resistant to transformation in vitro.

The characteristics of the HaCaT clearly document that spontaneous transformation of human adult keratinocytes can occur in vitro and is associated with sequential chromosomal alterations, though not obligatorily linked to major defects in differentiation *(Boukamp, 1988).*

2.3.1 Keratinocytes and UVB exposure

UV radiation generates reactive oxygen species, induces protein oxidation and DNA damage that subsequently leads to skin inflammation, photo aging and contributes to skin cancers.

Keratinocytes respond to UV radiation by producing and secreting proinflammatory cytokines, such as interleukin (IL)-1a and tumour necrosis factor-α (TNF-α). TNF-α contributes to the induction of apoptosis of keratinocytes after UV exposure. Moreover, TNF-α has been shown to mediate tumour promotion and to act as a paracrine inhibitor of human melanocyte proliferation and melanogenesis *(Garcin et al., 2009).*

Several of the TNF-α proinflammatory and cytotoxic responses in the skin can be inhibited by the alpha-melanocyte stimulating hormone (α-MSH). This is a tridecapeptide resulting from the proteolytic cleavage of proopiomelanocortin and is expressed both in the central nervous system and peripheral tissues including the skin.

α-MSH binds to the melanocortin-1 receptor (MC1R) on melanocytes to stimulate pigmentation and modulate various cutaneous inflammatory responses. MC1R expression is not restricted to melanocytic cells and may be induced in keratinocytes after UVB exposure *(Garcin et al., 2009).*

2.4 G protein coupled receptors (GPCRs)

GPCRs are types of receptors that are used in transmission and communication between cells. This type of cell is able to capture a signal from outside the body. Then via a cascade of intracellular mechanisms these cells are able to activate enzymes and commence gene expression. Cell response to incoming signals is the after-effect of this process *(Alberts et al., 2005)*

GPCRs are remarkably versatile signalling molecules. The members of this large family of membrane proteins are activated by a spectrum of structurally diverse ligands, and have been shown to modulate the activity of different signalling pathways in a ligand specific manner *(Kobilka, 2007).*

A variety of bioactive molecules (including biogenic amines, peptides, lipids, nucleotides, and proteins) modulate GPCR activity to affect regulation of essential physiological processes (e.g. neurotransmission, cellular metabolism, secretion, cell growth, immune defence, and differentiation). Thus, many important cell recognition and communication processes involve GPCRs.

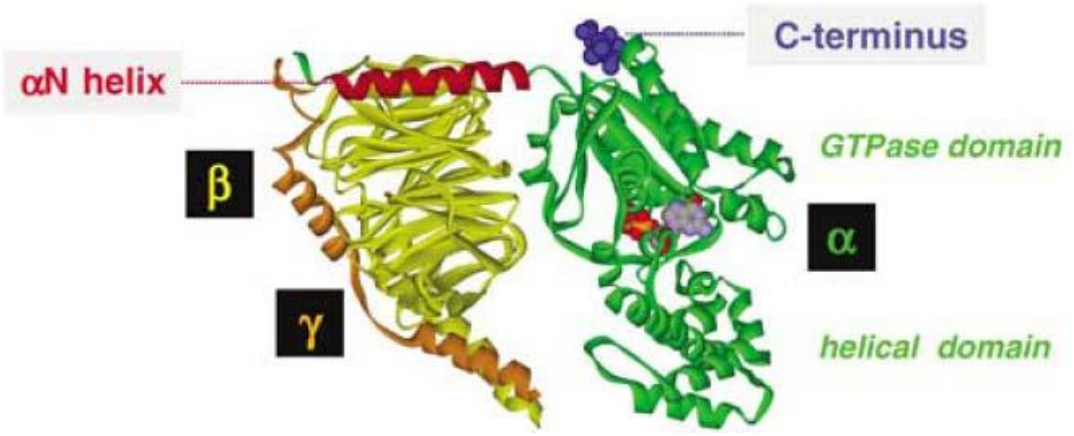
Due to their mediation of numerous critical physiological functions, GPCRs are involved in all major disease areas, including cardiovascular, metabolic, neurodegenerative, psychiatric, cancer, and infectious diseases (Goddard and Abrol, 2007).

2.4.1 Structure of GPCR

Signal molecules binding to the GPCR have different structures and functions. Conversely, all GPCRs analysed to date have the same structure *as seen in* **Figure 4.**

Thus all GPCRs have seven-transmembrane (TM) domains, connected by extracellular (ECL) and intracellular (ICL) loops *(Millar and Newton, 2010).*

Figure 4: ***Structure of G-protein coupled receptor*****.**

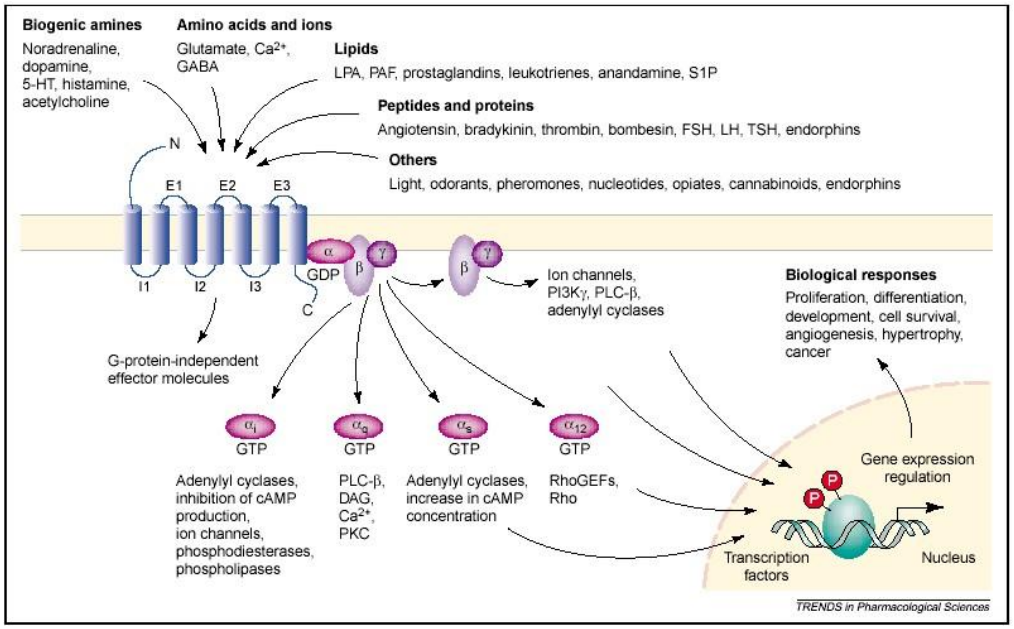


Three-dimensional x- ray crystal structure of GPCR (rhodopsin) consisting of α, β and γ subunits. The α subunit C-terminus residues convey GPCR specificity, shown in blue. The red N-terminus helix is required for binding of Gα to other subunits.
(adapted from: http://openwetware.org/wiki/BIO254:Gprotein – image from Milligan, 2006)

Based on sequence similarity within the 7 TM segments, these receptors can be clustered into 5 families: the largest rhodopsin family (light activated photoreceptor protein in the retina of vertebrates), and smaller family – adhesion, fizzled/taste, glutamate and secretin *(Kobilka, 2007).*

The rhodopsin-like family (or Family A) is by far the largest of these families and consists of rhodopsin, adenosine, melanocortin, neuropeptide, olfactory, chemokine, and melatonin receptors, amongst others. The secretin-like family (or Family B) consists of 25 members, including the receptors for the gastrointestinal peptide hormone family (secretin, glucagon, vasoactive intestinal peptide and growth hormone-releasing hormone), calcitonin, and parathyroid hormone, as well as corticotrophin-releasing hormone receptors. The metabotropic receptor family (or Family C) is the smallest family and consists of the GABA B receptor, the calcium-sensing receptor, and some taste receptors (see **Figure 5**) *(Lattin et al., 2007).*

Figure 5: ***Stimulation of G protein coupled receptor and biological responses***



Different signal molecules stimulate GPCR and the α subunit after binding GDP to GPT, reacting with different effectors, which results in different biological responses.
(adapted from: http://www.ibibiobase.com/projects/db-drd4/G_protein.htm)

2.4.2 Stimulation and activation of GPCR

After the signal molecule binds to the 7 TM receptor, conformation change and change of ICL is caused. Consequently, it results in the integration with GTP-binding proteins (G protein) situated on the intracellular side of cytoplasmatic membrane.

There exist numerous variants of G-proteins. For each of these variants there are typical specific groups of receptors. In any case, every type of G-protein has the same structure. It is composed of three subunits – α, β and γ *(Alberts et al., 2005).*

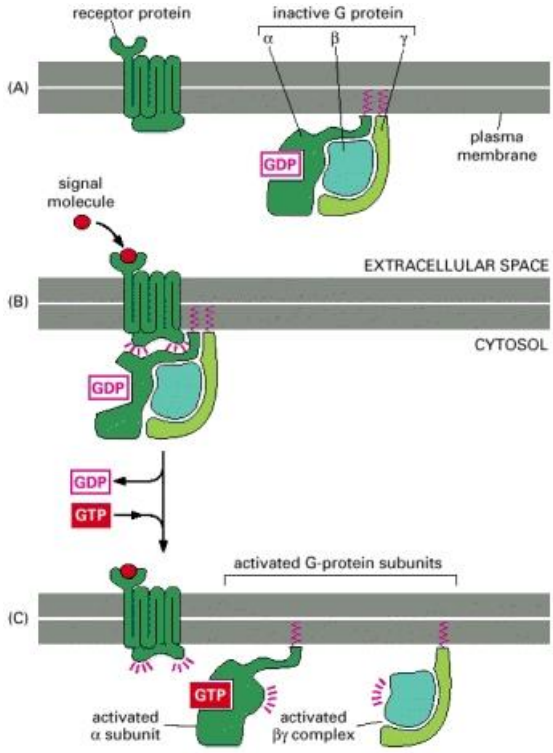
As shown in **Figure 6,** guanosine diphosphate (GDP) is in an inactive state bound to the α subunit and the G-protein is at rest. After the binding of extracellular ligand to the receptor, the receptor comes to the connection with the G-protein. This connection causes the conversion of GDP to guanosine triphosphate (GTP).

The conversion of GDP to GTP leads to division into two separate parts. One of these parts is an active subunit α with GTP and the second part is an inactive βγ komplex. These two independent units are capable of free diffusion

along the membrane. Moreover, the units can integrate with a specific place in the plasma membrane and then pass a signal to another destination.

The intensity and duration of the signal depends on the length of the α and βγ subunits. The α subunit has its own GTPase activity which influences the duration of signal. Subsequently, the GTPase activity causes the hydrolyzation of GTP to GDP. This backward change again evokes the binding of α subunit to βγ. This whole process, from activation to deactivation takes only a few seconds *(Alberts et al., 2005).*

Figure 6: ***Stimulation and activation of G protein coupled receptor***



(a)*GPCR without stimulation doesn't bind with G protein and it is at rest.*
(b)*After binding with the signal molecule it causes the establishing of GPCR with the G protein and GDP is converted to GTP.*
(c)*Gα subunit is dissociated out of Gβγ. These single activated subunits mediate signals and target either enzymes or ion channels.*
(adapted from: Albets et al., (2005) – page 494)

2.4.3 Desensitization of GPCR

As stated before GPCRs play a key role in controlling hormonal regulation of numerous second-messenger pathways. However, following agonist activation most GPCRs rapidly lose their ability to respond to hormones. For many GPCRs, this process is commonly referred to as desensitization *(Krupnick and Benovic, 1998).*

The uncoupling of GPCRs from their cognate heterotrimeric G proteins provides an essential physiological “feedback” mechanism that protects against both acute and chronic over stimulation of receptors *(Ferguson, 2007)*.

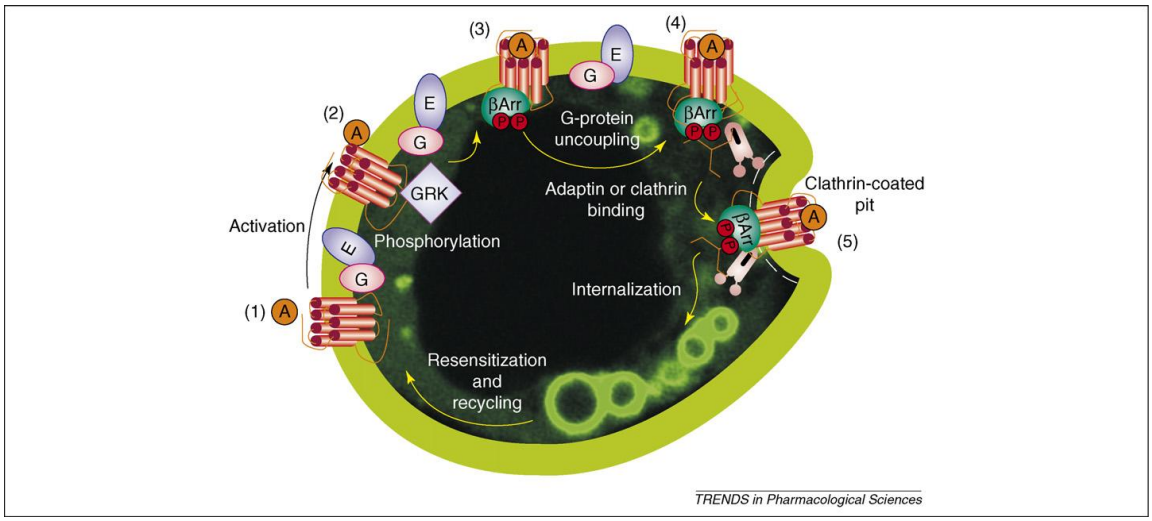
Classically, heterotrimeric (G proteins) are recruited to the activated conformation of 7TMRs. Only two other families of protein have this remarkable characteristic: G-protein coupled receptor kinases (GRKs) and β-arrestins. These two protein families have long been known to have a central and coordinated role in the “desensitization” of G protein activation by 7TMRs. In addition, GRKs and β-arrestins are involved in an increasing number of interactions with non-receptor proteins, broadening the variety of their cellular functions *(Reiter and Lefkowitz, 2006).*

GRKs recognize and phosphorylate only the agonist-activated GPCR conformation, thereby promoting the association of cytosolic cofactor proteins – arrestins *(Ferguson, 2007)*.

As seen in **Figure 7**, desensitization of GPCR occurs via a multistep process. GRKs phosphorylate the ICL and/or carboxyl terminal tail of the receptor, a process that enhances the affinity of the receptor for binding of cytosolic arrestin proteins. Subsequent binding of phosphorylated receptors by arrestins sterically inhibits interaction of the receptor with the G protein. Thus, agonist-induced phosphorylation of GPCR by a GRK, followed by binding of arrestins, efficiently prevents further coupling of the receptor to its G protein, thereby reducing or preventing receptor signalling.

Finally, the GRK-arrestin system promotes clathrin-mediated internalization of inactivated receptors to endosomal compartments for subsequent degradation or resensitization *(Yang and Xia, 2006).*

Figure 7: ***Desensitization of GPCR***



(1)*After the activation comes, during phosporylation, binding to GRK* ***(2)****, and followed by binding of beta arrestin* ***(3)****. Then GPCR uncoupling* ***(4)*** *and beta arrestin is clathrin-coated, this brings about internalization and resensitization.*
(adapted from: Ferguson, 2007)

2.5 G protein receptor kinase (GRKs)

GRKs were initially identified as a family of seven serine/threonine protein kinases. These kinases phosphorylate and regulate agonist-occupied or constitutively active GPCR. GRK family members can be subdivided into three main groups based on sequence homology: visual GRK subfamily (GRK1-also known as rhodopsin kinase and GRK7- termed also cone opsin kinase). This first group forms one distinct sub-group that is only found in retina cells. Another group is divided into two subgroups. The first subgroup is the β-adrenergic or non-visual receptor kinase subfamily: GRK2=βARK1 and GRK3=βARK2. The second and last group of GRKs is the GRK4 subfamily (GRK4, GRK5 and GRK6). GRK4 is predominantly found in the testes, to lesser extent, in some brain regions (cerebelum), testes and the kidneys. GRK2, 3, 5 and 6 are ubiquitously expressed in mammalian tissues (Reiter and Lefkowitz, 2006; Yang and Xia, 2006; Penela et al., 2010).

2.5.1 Structure of GRKs

GRKs share a common structural architecture with a well-conserved, central catalytic domain, similar to that of other serine–threonine kinases,

flanked by an N-terminal domain and a variable-length carboxyl-terminal domain. The N-terminal domain is important for receptor recognition, for intracellular membrane anchoring and it also contains an RH domain (regulator of G protein signalling homology domain) *(Ribas et al., 2007).*

The C-terminal domain of the GRKs contributes to their sub cellular localization and agonist-dependent translocation by favouring their interaction with lipids and other membrane proteins. The C-terminus of GRK2 and 3 contains a homology domain (PH) with binding sites for the membrane phospholipid PIP2 and free Gβγ subunits

Finally, GRKs are substrates for different kinases, which contribute to modulate kinase activity, localization and stability *(Ribas et al., 2007).*

2.5.2 GRKs and pathological effects in epithelial cells

GPCRs form the largest family of cell surface receptors and defects in GRK function have the potential to affect GPCR-stimulated biological responses in many pathological situations. Furthermore, the regulation of GRK levels in opiate addiction, cancers, psychiatric diseases, cystic fibrosis and cardiac diseases is discussed. Both transgenic mice and human pathologies have demonstrated the importance of GRKs in the signalling pathways of rhodopsin, β-adrenergic and dopamine-1 receptors. The modulation of GRK activity in animal models of cardiac diseases can be effective in restoring cardiac function in heart failure and opens a novel therapeutic strategy in diseases with GPCR disregulation *(Yang and Xia, 2006).*

Much new information regarding the phosphorylation and regulation of GPCR by GRK2 and GRK3 and their role in GPCR signalling has been revealed during the past few years. More recent studies have started to indicate roles for GRK4, GRK5 and GRK6, both in transfected cell lines and in primary cells. However, it remains to be established whether the multiplicity of GRKs is related to the specificity or differential regulation of GPCR signalling or indeed another, yet to be defined, function. The association of particular GRKs within receptor signalling, trafficking and switching is a key area of current and future investigation *(Yang and Xia, 2006).*

A potential role for GRK2 in epithelial cell migration was recently investigated. GRK2 promotes changes in actin cytoskeleton and paxillin localization consistent with enhanced focal adhesion turnover and higher cell motility. Moreover, GRK2 promotes increased migration toward fibronectin in different epithelial cell line models and in fibroblasts. These effects were independent of GRK2 kinase activity, as they were also observed upon expression of a catalytically inactive mutant. Therefore, and in contradistinction to immune cells, increased GRK2 expression facilitates migration towards fibronectin and GRK2 down-regulation impairs migration *(Penela et al., 2010).*

Altogether these results suggest that altered GRK2 expression in specific tumour cells may affect migration in response to particular stimuli and play a

role in carcinogenesis. This hypothesis is further supported by the observed cooperation of GRK2 with known oncogenes in „in vitro" transformation assays and by the emerging role of GRK2 in cell cycle progression (see below). A detailed characterization of GRK2 expression levels in different types of tumours and further insight on the effects of altered GRK2 expression in tumour progression are needed to further define its role in this process *(Penela et al., 2010)*.

2.6 Beta arrestin

Arrestins are a class of soluble proteins that function in concert with GRKs to stop or arrest intracellular signalling. In the continued presence of stimulus, arrestins ensure that each activated GPR is turned off first before reinitiating signal transduction. The prototypic arrestin is a protein, originally called S-antigen, which was first discovered as a causative antigen of experimental autoimmune uveitis, a degenerative eye disease. Retinal S-antigen was subsequently demonstrated to be the 48-kDa protein regulating light-dependent signal transduction in rod photoreceptor cells (hence its name - visual arrestin, also referred to as arrestin) *(Krupnick and Benovic, 1998)*.

The arrestins constitute a four-member family. The expression of arrestin 1 and arrestin 4 (x-arrestin) is restricted to retinal rods and cones, respectively. By contrast, arrestin 2 and arrestin 3 (better known as β-arrestin 1 and β-arrestin 2) are expressed ubiquitously *(Reiter and Lefkowitz, 2006).*

The light-dependent binding of arrestin to rhodopsin is highly enhanced by rhodopsin phosphorylation. Arrestin is soluble in the dark but associates with ROS disk membranes when rhodopsin absorbs light and is phosphorylated by rhodopsin kinase.

While rhodopsin and arrestin – specifically the β – arrestin 2 have served as useful model systems, agonist specific desensitization is a general phenomenon that has been observed for many GPRs *(Krupnick and Benovic, 1998).*

2.6.1 Beta arrestin and UVB

β-arrestin 2, one of the well-studied GPCRs, is ubiquitously expressed in the epidermis and function to maintain homeostasis in normal physiological settings as well as pathological states in the epidermis.

UV response in mammalian cells is mediated through the activation of several transcription factors, including nuclear factor kappa B (NF-κB) *(Luan et al., 2005).*

NF-κB activation is an important mechanism of mammalian UV response to protect cells. UV-induced NF-κB activation depends on the casein kinase II phosphorylation of α subunit of the NF-κB inhibitor (IκBα) at a cluster of C-terminal sites. But how it is regulated remains unclear. *Luan et al.(2005)*

demonstrated that β-arrestin 2 can function as an effective suppressor of UV-induced NF-κB activation through its direct interaction with IκBα. CK2 phosphorylation of β-arrestin 2 blocks its interaction with IκBα and prevents its suppression of NF-κB activation, indicating that the β-arrestin 2 phosphorylation is critical. Moreover, stimulation of β2-adrenergic receptors, a representative of G-protein-coupled receptors in epidermal cells, promotes dephosphorylation of β-arrestin2 and its suppression of NF-κB activation.

Consequently, the β-arrestin 2 suppression leads to promotion of UV-induced cell death, which is also under regulation of β-arrestin2 phosphorylation. Thus, β-arrestin 2 is identified as a phosphorylation-regulated suppressor of UV response and this may play a functional role in the response of epidermal cells to UV (*Luan et al., 2005).*

3 AIM OF WORK

The aim of my work was to analyze the effect of UVB radiation on GRKs and β-arrestin expression in HaCaT cells. Furthermore, we compared an *in vitro* model (HaCaT cells) with an *in vivo* model (human epidermis) in which we wanted to observe possible modulations of GRKs and β-arrestin 1.

The goals of the work were the following:

1) Review literature about UVB, GPCR, GRKs and β-arrestin.

2) Practice the methods: SDS-PAGE, Western blot and Coomassie blue staining.

3) Measure the viability of HaCaT cells by MTT test.

4) Determine the influence of UVB radiation on the GRKs and β-arrestin expression
   - in vitro – HaCaT
   - in vivo – epidermis

5) Optimization and comparison of different protocols for epidermis extraction from skin.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Laboratory equipment

Bio 1D image analysis software	Vilber Lourmat
Centrifuge Sigma 2K15	Biobloc Scientific
Enhanced chemiluminescence (ECL) substrate solution	Bio-Rad
Eppendorf precision pipettes (2.5, 20, 200, 1000 µl)	Eppendorf
Flasks (75 cm^2, 175 cm^2)	Schott
Humidified cell culture incubator (37°C, 5% CO_2)	SANYO
Laminar air flow hood	LABOVER
Mini-Protean 3 polyacrylamide electrophoresis	Bio-Rad
Nitrocellulose membranes (0.2 µm)	Amersham
Nitrogen Tank Chronos	Arpege 140
Optic microscope Olympus CK2	ULWCD
Pipet-aid automatic pipette	Eppendorf
Pipettes	Eppendorf
Power supply	Bio-Rad
Refrigators (4°C, -20°C, -80°C)	Liebherr
Spectrofotometr	Genesys
Thermoblock	Eppendorf
UVB lamp - Waldmann 800k light source	Herbert Waldmann
VICTOR microplate reader	Life Sciences Inc
Vortex shaker	Heidolph
Water bath	Julabo Labortech
Western blot apparatus	Bio-Rad
Whatman Fitrerpaper	Whatman

4.1.2 Chemicals

4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride
Acetic acid
Acrylamide-bis acrylamide solution 40%
Ammonium persulfate (APS)
Bromophenol blue
BSA fraction V
Dithiothreitol (DTT)
DMEM
FBS
Glycerol

Hydrochloric acid
L-glutamine
Low-fat non-supplemented milk powder (Regilail)
Methanol
MTT solution
N, N, N', N'-tetramethyl-ethylenediamine (TEMED)
PBS
penicillin-streptomycin
Phosphate buffer saline (PAA laboratories)
Ponceau S
RNA*later*® (Sigma)
SDS
Sodium chloride
Solution A, B (Lumigen)
Thermolysine
Trichloroacetic acid
Tris-hydroxymethyl-aminomethane
Triton X-100
trypsin-EDTA
Tween 20
β-Mercaptoethanol

The majority of chemicals were acquired from Sigma-Aldrich.

4.1.3 Antibodies

The antibodies entered in **Table 1** were used in this work.

Table 1: ***List of antibodies used in this work***

Type	Specifity	Dilution	Manufacturer
Primary antibody	Anti- GRK1 Anti-GRK2 Anti-GRK6 Anti-β arrestin 1	1:1 000	Abcam
Secondary antibody	The anti-mouse IgG-HRP The anti-rabbit IgG-HRP	1:4 000	Cell Signalling Technology

4.1.4 Buffers

DMEM medium

FBS	10 %
L-glutamine	2mM
Penicillin	25 U/ml
Streptomycin	25 µg/ml

Lysis buffer (Triton high protection)

HEPES acid	50 mM
Triton X-100	1 %
EDTA	1 mM
Sodium floride	10 mM
Pyrophosphate	30 mM
Vanadate	1 mM
Bacitracine	1 mg/ml

Coomassie blue (for 1 l – filtrated with Whatman paper)

Coomassie blue R250	1 g
Ethanol	450 ml
Ice acetic acid	50 ml

TBS buffer 10X

Tris Base	24,2 g
Sodium chloride	80 g
Distilled water	qsp 1 l

TTBS buffer

TBS buffer 10X	100 ml
Tween 20	5 ml
Distilled water	qsp 1 l

Blocking buffer

Non-fat milk	2,5 g
BSA	0,5 g
TTBS buffer	qsp 50 ml

Western blot buffer 10X

Tris base	25 mM
Glycine	192 mM
Distilled water	qsp 1 l

Migration buffer

Western-Blot buffer 10X	100 ml
SDS 10 %	10 ml
Distilled water	qsp 1 l

Transfer buffer

Western-Blot buffer 10X	100 ml
Methanol	200 ml
SDS 10 %	10 ml
Distilled water	qsp 1 l

Buffer pH 8,8 (Tris 1,5 M, SDS 0,4%)

Tris Base	18,7 g
SDS 10 %	4 ml
Distilled water	qsp 100 ml

Buffer pH 6,8 (Tris 0,5 M, SDS 0,4%)

Tris Base	6 g
SDS 10 %	4 ml
Distilled water	qsp 100 ml

Stacking gel (4%)

Distilled water	3,16 ml
Buffer pH 6,8	1,26 ml
SDS 10 %	50 µl
Acrylamide 40 %	0,5 ml
APS 10 %	50 µl
TEMED	5 µl

Running gel (10%)

Distilled water	9,69 ml
Buffer pH 8,8	5 ml
SDS 10 %	200 µ
Acrylamide 40 %	5 ml
APS 10 %	200 ml
TEMED	10 µl

6X SDS Sample buffer for denaturation of samples before SDS-PAGE

1M Tris/Hcl (pH 6,7)	0,375 M
Glycerol	60 %
SDS	12 %
DTT	0,6 M
Bromophenol blue	0,06 %
Distilled water	qsp 10 ml

TE buffer

Tris-HCl pH 7,5	40 mM
EDTA	10 mM
AEBSF	1 mM
Natrium floride	10 mM
Pyrophosphate	30 mM
Vanadate	1 mM
Bacitracine	1 mM

TE-NP40 buffer

TE buffer	
NP-40	0,5 %

TUDTT buffer

Tris-HCl pH 6,7	35 mM
Urea	8 M
DTT	50 mM
Glycerol	5 %
AEBSF	1 mM
Sodium floride	10 mM
Pyrophosphate	30 mM
Vanadate	1 mM
Bacitracin	1 mM

Stripping buffer

SDS	2 %
Tris 1M	60 mM
β-mercaptoethanol	100 mM

Buffer 5X

Tris-HCl ph 8,3	250 mM
Kalium chloride	375 mM
Magnesium chloride	15 mM
DTT	2 µl

Coloration solution

Ethanol	45 %
Acetic acid	5 %
Coomassie blue R-250	

Decoloration solution

Ethanol	5 %
Acetic acid	7,5 %

4.2 Methods

4.2.1 Cell culture

The HaCaT cell line was obtained from N.E. Fusenig (Institute of Biochemistry, German Cancer Research Center, Heidelberg, Germany). The cells were cultured in DMEM (see section **4.1.4**) and maintained at 37°C in 5% CO_2 in a humidified environment. After each passage, the cells were diluted 10 times.

4.2.2 Cell lysis

The HaCaT cells were seeded ($1x10^6$ cells per 100 mm in Petri dishes) with PBS + 1% FBS. A part of them was exposed to a dose of 30 mJ/cm² UVB radiation and the other part was left on the table exposed (condition « sham-irradiated »). The cells were irradiated with a Waldmann 800k light source. After that, the medium of cells was replaced by DMEM without serum and the cells were incubated for different incubation times (3, 5, 7, 9, 17 and 24 hours).

The UVB-irradiated cells and non-irradiated controls were lysed by 500 µl of lysis buffer Triton high protection buffer (see section **4.1.4**) and incubated for 30 min in ice.

Total lysates were centrifuged (12 000 g, 4°C, 30 min) and the protein concentration of each sample was measured with a Bradford test.

4.2.3 Sequential protein extraction of epidermis

Pieces of skin from a plastic surgery (Clinique St Jean, Montpellier) were placed in DMEM at 4°C overnight. The next day, adipocyte tissue was eliminated and the skin was cut into small pieces (1 cm^2). To conserve tissue integrity, we placed the skin in RNA*later*®. Then we used 2 types of preparation of the epidermis (see **Figure 8** and **Figure 9**):

4.2.3.1 Preparation of epidermis without UVB irradiation (Figure 8)

Fresh epidermis (frozen skin)

Pieces of skin were frozen in liquid nitrogen with RNA*later*®. Before using them, we took the pieces of skin out of the liquid nitrogen and thawed them in a water bath (40°C, 5 min). We then separated the epidermis of the skin after an incubation in thermolysin (0,5 mg/ml in PBS) for 1,5 – 2 hours at 37°C in 5% CO_2 in humidified environment and extracted the protein.

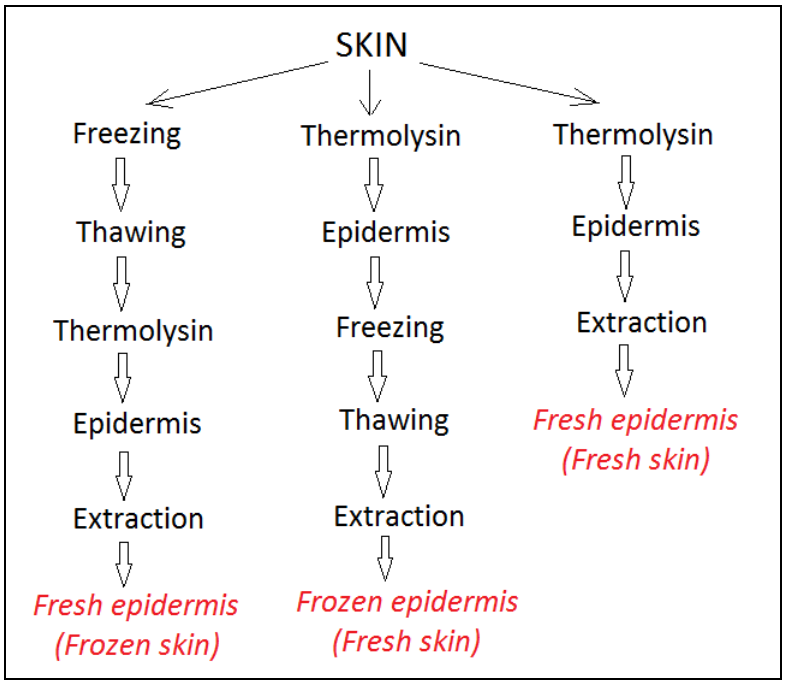
Frozen epidermis (fresh skin)

We put pieces of fresh skin in thermolysin (0,5 mg/ml in PBS) for 1,5 - 2 hours at 37°C in 5% CO_2 in a humidified environment. Then we separated the epidermis from the dermis, replaced the epidermis in RNA*later*® and saved them in liquid nitrogen. Before use, the epidermis was thawed in a water bath (40°C, 5 min) and we then extracted the protein.

Fresh epidermis (fresh skin)

We had pieces of fresh skin and we placed them in thermolysin (0,5 mg/ml in PBS) for 1,5 - 2 hours at 37°C in 5% CO_2 in a humidified environment. Then we separated the epidermis from the dermis and carried out the extraction. We didn't carry out the step of freezing them in liquid nitrogen.

Figure 8: ***Preparation of epidermis without UVB irradiation***

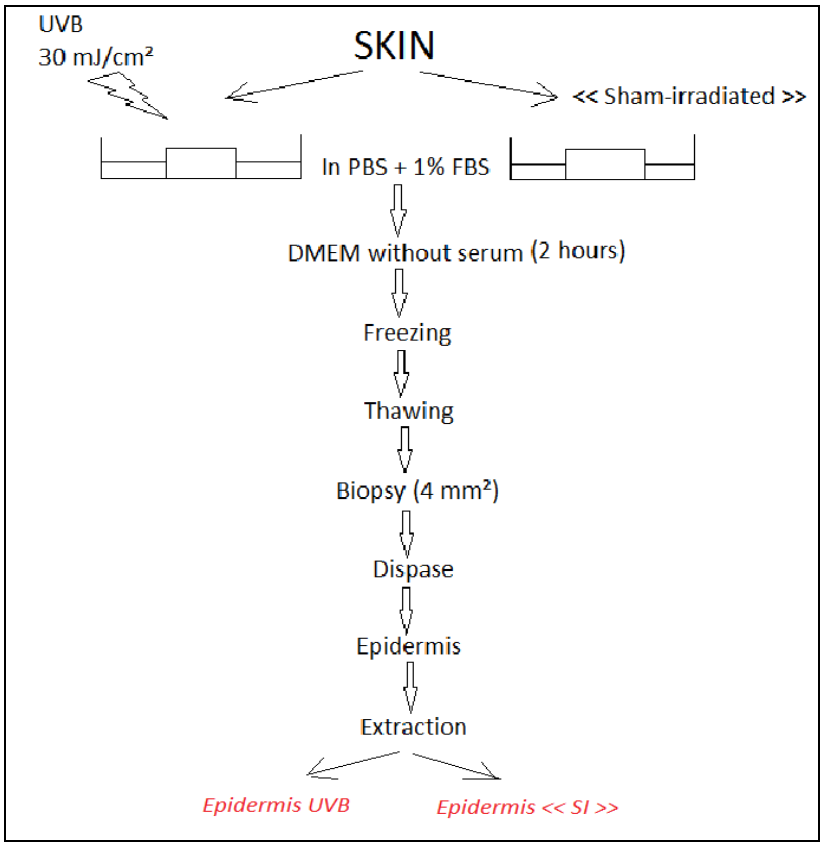


As we can see in this figure, we prepared 3 types of epidermis – fresh epidermis (frozen skin), frozen epidermis (fresh skin) and fresh epidermis (fresh skin).

4.2.3.2 Preparation of epidermis with UVB irradiation (Figure 9)

The skin was seeded (1cm^2 per 100 mm in Petri dishes) with PBS + 1% FBS. One part of it was exposed to UVB radiation (30 mJ/cm²) and the second part was left on the table exposed (condition «sham-irradiated»). After 2 hours in DMEM without serum, we saved the pieces of skin in liquid nitrogen with RNA*later*®. Before use, we thawed the skin in a water bath (40°C, 5 min) and then made a biopsy (4 mm²). Each sample was incubated with 250 µl of dispase (2 mg/ml) at 37°C in 5% CO_2 in a humidified environment for 1 hour and 30 minutes. Thereafter, we separated the epidermis from the dermis and carried out the extraction.

Figure 9: ***Preparation of epidermis with UVB irradiation*****.**



The first sample of skin was exposed to UVB radiation and the second sample "SI"= "sham-irradiated" was left on the table for this time. Following adjustment it was the same for both samples.

4.2.3.3 Sequential protein extraction

We carried out sequential protein extraction according to the protocol described in the publication of *Simon et al.(1997).*
The epidermis was homogenized three times in ice with 600 µl of hypotonic TE buffer and TE containing NP-40 buffer (see section **4.1.4**). The obtained pellets were divided into three parts which were extracted with 200 µl of TE buffer containing 4, 6 or 8 M of urea (TEU4, TEU6 and TEU8 respectively).

After extraction, the samples were centrifuged (15 000 g, 4°C, 15 min) and the pellets obtained with the TEU8 buffer were incubated with 200 µl of TUDTT buffer at 95°C for 30 min. Then the samples were centrifuged (15 000 g, 4°C, 15 min) and the protein concentration of each sample was measured with a Bradford test.

4.2.4 Bradford test

The Bradford protein assay is a simple procedure for determination of protein concentration in solution that depends upon the change in absorbance in Coomassie Blue G-250 upon the binding of protein *(Bradford et al., 1976).*

We prepared BSA (Bovine Serum Albumin) dilutions of known concentrations (0, 1, 2, 3, 4, 6, 8 and 10 µg/ml) and 2 µl of each unknown sample. We added 800 µl of Coomassie blue diluted 5 times (see section **4.1.4**) in each tube. After an incubation of 15 min at room temperature we measured the absorbance at 595 nm with a spectrophotometer.

After, the Bradford test samples were denaturated with blue 6X SDS sample buffer (see section 4.1.4) at 95 °C for 5 min.

4.2.5 SDS-polyacrylamide electrophoresis (SDS-PAGE) and Western blotting

4.2.5.1 SDS-PAGE

Western immunoblotting after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used method for the study of proteins. Proteins are separated according to their molecular mass in the presence of SDS in polyacrylamide gels by electrophoresis.

SDS is responsible for the denaturation of proteins and their negative charge whereby they move to an anode. As smaller proteins move more easily across the gel during migration, proteins are separated according to their molecular weight. The migration of proteins is influenced by the porosity of the gel, which depends on the concentration of acrylamide and bis-acrylamide in the running gel.

Modern SDS polyacrylamide gels consist of two gel layers: the uppermost "top/stacking" gel (see section **4.1.4**) is less concentrated and allows for the concentration during the earliest part of the migration of the protein samples into a thin protein band which is thereafter resolved in the "separating/resolving/running gel" during electrophoresis.

A 25 µg dosage of protein lysates/well was loaded and electrophoresis was performed at 90 V for 30 min and at 200 V for 30 min in a migration buffer for 1 hour and 30 minutes. Electrophoresis was stopped when the bromophenol blue front arrived at the bottom of the gel.

4.2.5.2 Western Blot

After migration, the proteins were transferred to nitrocellulose membranes at 85 V for 1 hour and 30 minutes in a transfer buffer (see section **4.1.4**). Transfer to nitrocellulose membrane permits consequent immunodetection of specific protein bands using specific antibodies.

4.2.5.3 Immunoblotting

Blots were blocked in a blocking buffer (see section **4.1.4**) overnight at 4°C. The next day, the blots were incubated with different primary antibodies (see section **4.1.3**) at room temperature for 3 hours. Primary antibody binding was detected by incubation with specific anti-rabbit or anti-mouse secondary antibodies linked to HRP for 1 hour.

The blots were analyzed with the Bio 1D image analysis software and the results were expressed as relative optical densities.

4.2.5.4 Stripping of membranes

Stripping is the term used to describe the removal of primary and secondary antibodies from a western-blot membrane. Stripping is useful to investigate more than one protein on the same blot - for instance, a protein of interest and a loading control. When probing for multiple targets, stripping and re-probing a single membrane instead of running and blotting multiple gels have the advantage of saving samples, materials, and time.

It is not advisable to make quantitative comparisons of targets probed before and after stripping since the procedure removes some sample protein from the membrane. For the same reason, a stripped membrane should not be probed to demonstrate the absence of a protein. We use this procedure for comparison of results with β-actin, as an internal control. Membranes were incubated in stripping buffer (see section **4.1.4**) for 45 minutes at 60 °C in a water bath with a shaker. After that, the membranes were washed with TTBS twice for 30 minutes. After that we were able to add new primary antibodies.

4.2.5.5 Staining Protein Gels with Coomassie Blue

This visualization of protein is useful to determine if proteins have migrated uniformly and evenly.

After migration, we putted the gels in a coloration solution (see section **4.1.4**) filtered by Whatmann paper for 20 minutes at 55°C. After coloration, the gels were washed 3 times for 20 min at 55 °C in a decoloration solution (see section **4.1.4**). Decolorized gels were left in distilled water overnight. The next the day, the gels were equilibrated in a drying solution and were dried in a GelAir drying system.

4.2.6 MTT test

MTT testing of cellular viability is a rapid colorimetric assay of the quantification of viable cells. This colorimetric assay was originally described by *Mosmann et al., (1983)* and is a useful method for the measurement of in vitro cytotoxicity, viability and cell proliferation.

The reactive substance is the salt of tetrazolium MTT (3-(4, 5-dimethylthiazol-2-y1) 2, 5-diphenyl tetrazolium bromide). It is a yellow substance which is oxidized by succinate deshydrogenase in mitochondria of live cells to a blue colour formazan. In aqueous solution, formazan creates crystals which can dissolve in organic solvent (in our case DMF – N, N-dimethylformamide). The intensity of this coloured solution is directly proportional to the number of viable target cells. Absorbance of the solution and thus the numbers of viable cells growing in microplate wells can be read by an automatic microplate scanning spectrophotometer, which offers major advantages in speed, simplicity, cost and safety.

In our case HaCaT cells were cultivated in DMEM containing 10% FBS, 2 mM L-glutamine, 25 U/ml penicillin, 25 µg/ml streptomycin and maintained at 37°C in 5% CO_2 in a humidified environment. After that, the number of cells was calculated with a Thoma counting chamber and diluted at 1 000 000 cells/ml in PBS containing 1% FBS.

A part of the HaCaT cells were irradiated by different doses of UVB (as seen in Table 2). The second part was not irradiated (non-irradiated control). After that the cells were centrifuged (5 min, 1500 g) and then resuspended in the same volume of DMEM without serum and without phenol red. The cells were seeded in a 96-microplate (100 µl/well) and incubated for 18 hours at 37°C in 5% CO_2 in a humidified environment.

The next day, MTT solution was prepared - 5 mg/ml in DMEM without serum and without phenol red and filtrated (0,2 µm). MTT was added to the cells (50 µl/well) and was incubated until violet crystals appeared (at least 2 hours). After, the violet crystals were dissolved with 100 µl of MTT buffer (see

section **4.1.4**). The absorbance was measured at 570 nm and at 660 nm with a VICTOR microplate reader.

Table 2: ***Exposition times of cells for different doses of UVB***

UVB dose (mJ/cm²)	Exposition time (minutes)
30	1´22
60	2´44
90	4´06
150	5´11
300	10´22
395	14´00

4.2.7 RNA extraction

Sigma's Gen Elute Mammalian Total RNA MiniprepKit provides a simple and convenient way to isolate total RNA from mammalian cells and tissues. Protocols are provided for cells, tissues, and fibrous tissues. These protocols differ in their cell lysis and disruption conditions. For fibrous tissues the kit must be used with proteinase K to ensure effective cell disruption. Once the RNA is bound to the Gen Elute Binding Column, the purification procedure is the same for all starting materials.

The purified RNA is ready for reverse transcription and PCR, labelling and micro array analysis and other common applications. To effectively release RNA from fibrous tissues, it must be used with proteinase K.

The most difficult step in extracting RNA from fibrous tissue is completely disrupting the tissue. These tissues are polynucleate, have a low cell density, and contain an abundance of contractile proteins, connective tissue and collagen. Tissues with these characteristics typically give low RNA yields. To overcome these problems the protocol must be modified to include a proteinase K digestion to facilitate cell disruption. This procedure has been used for the isolation of total RNA from heart, skeletal muscle and skin. Up to 40 mg of tissue may be used per preparation. It is recommended to start with no more than 25 mg of tissue and then empirically determine the maximum amount of tissue that can be processed *(Protocol by Sigma-Aldrich).*

In our work, tissues were prepared in this way: We performed the extraction with epidermis which was saved in 0,5 ml of RNA in liquid nitrogen. We thawed them and made 4 types of biopsy:

a) m=4,16 mg
b) m=8 mg
c) m=15 mg
d) m=17 mg

Then we lysed tissue and inactivate RNase by adding 300 ml of the Lysis Solution/2-ME. We mixed and homogenized immediately until no visible pieces remained.

Then we removed 700 ml of the digested tissue into a Gen Elute Filtration Column (blue insert with a 2 ml receiving tube). We centrifuged the preparation at maximum speed (12 000–16 000 g) for 2 minutes. After that we transferred the filtrate to a new 2 ml micro centrifuge tube. Finally we repeated the step with the remainder of the digested tissue and pooled the filtrates.

We then added 1 ml of 70% ethanol to the filtered lysate. We mixed it by pipetting and put 700 ml of the lysate/ethanol mixture into a Gen Elute Binding Column (colourless insert with a redo-ring seated in a 2 ml receiving tube). We then centrifuged for 2 minutes. We discarded the flow through liquid but retained the collection tube. We returned the Pipette 250 µL of Wash Solution 1 into the column and centrifuged it at maximum speed for 2 minutes.

Then we mixed 50 µl of DNAse 1 with 350 µl of Tp digestion/ for 5 preparation and added 80 µl of mixture on the filter of the red column. We incubated for 15 minutes in TA and 250 µl of was solution 1. Then we centrifuged for 2 minutes. We transferred it to the red column in a new tube. We added 500 µl of wash solution 2. We again centrifuged for 2 minutes. We repeated the step of washing in solution 2 and centrifugation for 2 minutes. The binding column must be free of ethanol before eluting the RNA so we centrifuged again for 2 minutes to totally remove the ethanol. Then we transferred it to the red column in a new tube, added 50 µl of Elution solution and centrifuged 2 minutes. If more than 50 mg of RNA was expected, we repeated the elution with another 50 ml of the Elution Solution and collected both eluates in the same tube. Purified RNA was now in the flow-through eluate (~45 or 90 ml total) and was ready for immediate use or storage at –70°C. We kept the RNA on ice until it was thawed for use. Then we prepared RNA in a dose 4 µl of RNA + 9996 µl of Tris 10 mM pH 7,5.

4.2.8 Inverse transcription

The method of inverse transcription enables synthesis of isolated RNA to complete DNA (cDNA).

This synthesis requires the use of a DNA-dependent RNA polymerase (MMLV, Invitrogen), a reverse primer and a mixture of the four deoxyribonucleotides triphosphates: dATP, dGTP, dCTP, dTTP (dNTP, Invitrogen).

In our case, we used a poly-T primer that will bind specifically to the tail poly-A mRNA and thus allow the synthesis of cDNA from all mRNAs present in the sample. In practice, we mixed two micrograms of RNA with 1 µl of oligo-dT (Invitrogen) and 1 µl of dNTP then incubated it for 5 minutes at 65 ° C. Then we added 4 µl of buffer 5X (see section **4.1.4**) and RNA polymerase.

Then we adjusted the reaction mixture to 20 µl with DEPC water and incubated at 37°C for 50 minutes and then at 70°C for 5 minutes. The samples were stored at -80°C.

4.2.9 Quantitative PCR

Quantitative PCR (qPCR) allows quantification of starting amounts of DNA, cDNA, or RNA templates. qPCR is based on the detection of a fluorescent reporter molecule that increases as PCR product accumulates with each cycle of amplification. Fluorescent reporter molecules include dyes that bind double-stranded DNA *(protocol by Sigma-Aldrich).*

In our case we used fluorescent SYBR Green I. This fluorophore binds in the minor groove of the DNA double helix regularly (about 1 molecule every 10 base pairs) resulting in the emission of a fluorescence at 530 nm. Thus, the fluorescent signal emitted during the PCR reaction is proportional to the amount of synthesized DNA and the amplified fragment length.

The major drawback of this type of marker is it lack of specificity since it binds to any DNA sequence present in the reaction medium. The specificity of the amplified signal is monitored by analyzing the curves obtained at the end of fusion PCR which gives the melting temperatures of amplified products.

For each sample, an internal control was used. It allows normalization of the results *(Henri, 2010).*

qPCR exceeds the limitations of traditional end-point PCR methods by allowing either absolute or relative quantification of PCR product at the end of each cycle. This ability has greatly enhanced several areas of research including gene expression analysis and genotyping assays *(Protocol by Sigma-Aldrich).*

The cDNA of our samples was diluted five times before being used in qPCR. The protocol used is that described by Roche for the marker SYBR Green I (LightCycler ® Fast Start DNA MasterPlus SYBR Green I, Roche).

The primer used for MC1R determination is described in **Table 3.** The rest of the primers on request from *Pauline Henri, IBMM institute*.

Table 3: ***Primer for qPCR***

	sense primer	**reverse primer**
MC1R	TGTCGTCTTCAGCACGCTCTT	CGTACAGCACGGCCATGA

The PCR conditions were as follows:

Phase of initial denaturation: 95°C for 10 minutes

Amplification phase: 40 cycles in total, each consisting of the following phases: denaturation (95°C for 10 seconds), annealing (58°C for 10 seconds) and elongation (72°C for 10 seconds)

Melting curve: from 50°C to 95°C with a ramp of 0.1°C per second.

5 RESULTS

5.1. Modulation of GRKs and β-arrestin 1 by UVB in HaCaT cells *in vitro*

5.1.1 Effect of UVB irradiation on GRK2 and GRK6 in HaCaT cells *in vitro*

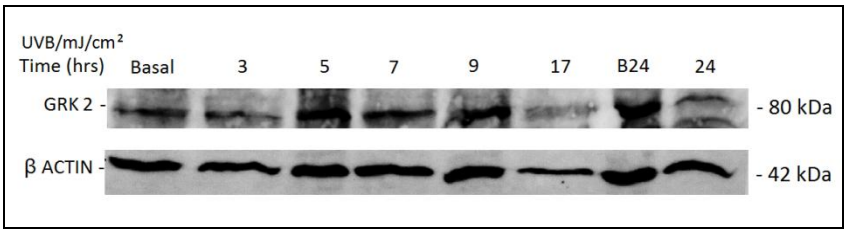
As GRK2 and GRK6 are expressed in melanoma cells and in normal melanocytes, we wanted to know if it was the case in the keratinocyte cell line HaCaT.

To investigate the modulation of GRK2 and GRK6 by UVB *in vitro*, HaCaT cells were exposed, in single measurement, to 30 mJ/cm^2 of UVB. After that, cells were incubated for different times (3, 5, 7, 9, 17, 24 hours). In parallel, we used a non-irradiated control (basal) and a “sham-irradiated” sample after 24 hours of incubation (B24). Cellular extracts of HaCaT wt (25 µg) were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with an anti-GRK2 antibody (see Figure 10) or an anti-GRK6 antibody (see Figure 11). As an internal control of the quantity of protein in each well, we used β-actin which has an ubiquitary expression in cells.

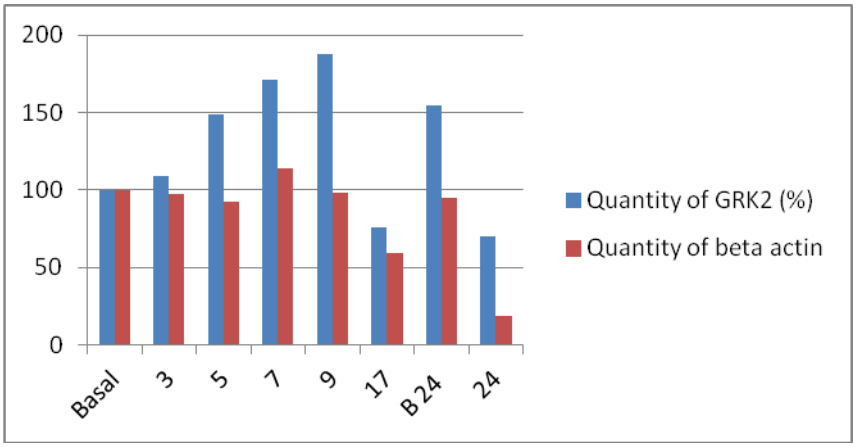
As seen in Figure 10 we observed a time-dependent increase of GRK2 expression between 5 hours and 9 hours after 30 mJ/cm2 of UVB in HaCaT cells (expression increased by 148,4% 5 hours after UVB, by 171,3% 7 hours after UVB and by 187,4% 9 hours after UVB). 17 hours after UVB irradiation, GRK2 expression decreased to its basal level. We also observed that β-actin was not homogeneous between wells. As we can see in Figure 11, GRK6 didn’t seem to be modulated by 30 mJ/cm2 of UVB in the keratinocyte cell line HaCaT.

Figure 10: ***Expression of GRK2 and β-actin proteins in UVB-irradiated HaCaT cells***

a)



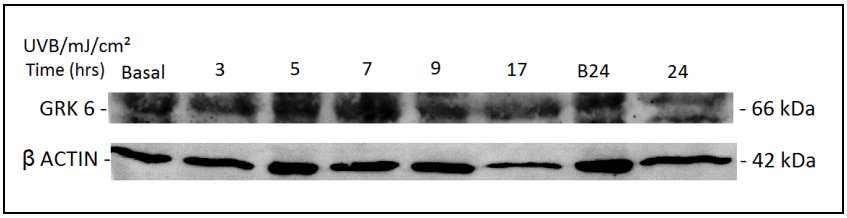
b)



a) *HaCaT wt were exposed to UVB radiation (30 mJ/cm²) and cells were incubated for 3, 5, 7, 9, 17 and 24 hours. In parallel, we used a non-irradiated sample (basal) and "sham-irradiated" sample after 24 hours of incubation (B24). Cellular extracts (25 µg/well) were resolved by SDS-PAGE electrophoresis, were transferred to nitrocellulose membranes and were probed with an anti-GRK2 antibody (see section* ***4.1.3****). Primary antibody binding was detected by incubation with an anti-rabbit secondary antibody linked to HRP. Membranes were stripped as described in the Materials and Methods section and were then reprobed with an anti-β-actin antibody.*

b) *Blots with GRK2 and β-actin were analyzed with the Bio 1D image analysis software and results were expressed as percentage (%) compared to basal normalized at 100 %.*

Figure 11: ***Expression of GRK6 and β-actin proteins in UVB-irradiated HaCaT cells***



HaCaT wt were exposed to UVB radiation (30 mJ/cm²) and cells were incubated for 3, 5, 7, 9, 17 and 24 hours. In parallel, we used a non-irradiated sample (basal) and "sham-irradiated" sample after 24 hours of incubation (B24). Cellular extracts (25 µg/well) were resolved by SDS-PAGE electrophoresis, were transferred to nitrocellulose membranes and were probed with an anti-GRK6 antibody (see section ***4.1.3****). Primary antibody binding was detected by incubation with an anti-mouse secondary antibody linked to HRP. Membranes were stripped as described in the Materials and Methods section and were then reprobed with an anti-β-actin antibody.*

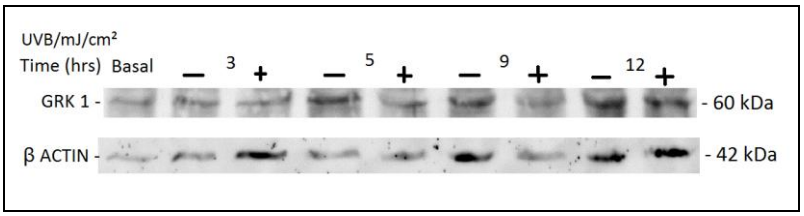
5.1.2 Effect of UVB irradiation on GRK1 and β-arrestin 1 in HaCaT cells *in vitro*

To investigate the modulation of GRK1 and β-arrestin 1 by UVB *in vitro*, HaCaT cells were exposed to 30 mJ/cm^2 of UVB. After that, cells were incubated for different times (3, 5, 9, 12 hours). In parallel, we used a non-irradiated control (basal) and "sham-irradiated" samples after 3, 5, 9 and 12 hours of incubation (UV). Cellular extracts of HaCaT wt (25 µg) were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with an anti-GRK1 antibody (see **Figure 12a**) or an anti-β-arrestin 1 antibody (see **Figure 12b**). As an internal control of the quantity of protein in each well, we used β-actin which has an ubiquitary expression in cells.

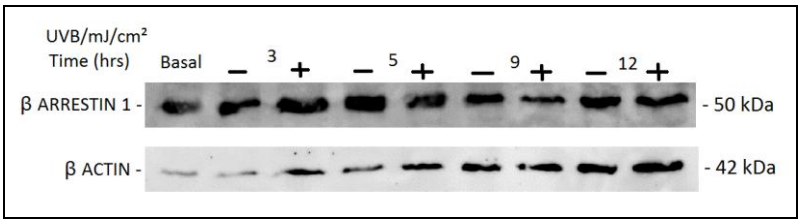
As we can see in **Figure 12**, GRK1 and β-arrestin 1 proteins are expressed in the keratinocyte cell line HaCaT. But it's difficult to conclude on the modulation of GRK1 and β-arrestin 1 expression because β-actin is heterogeneous between wells. In fact, we used a membrane stripping procedure, which can result in the loss of some proteins. Nevertheless, it would not seem that GRK1 and β-arrestin 1 are modulated by UVB in HaCaT cells.

Figure 12: ***Expression of GRK1, β-arrestin 1 and β-actin proteins in UVB-irradiated HaCaT cells.***

a)



b)

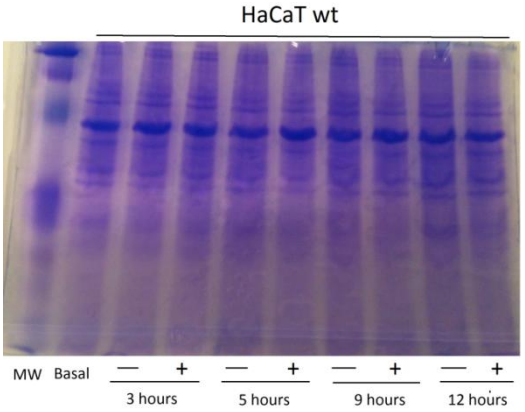


HaCaT wt were exposed to UVB radiation (30 mJ/cm²) and the cells were incubated for 3, 5, 9 and 12 hours. In parallel, we used a non-irradiated sample (Basal) and "sham-irradiated" samples after 3, 5, 9 and 12 hours of incubation (UV). ***a)*** *Cellular extracts (25 µg/well) were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with an anti-GRK1 antibody (see Table 1). Primary antibody binding was detected by incubation with an anti-mouse secondary antibody linked to HRP. Membranes were stripped as described in the Materials and Methods section and were then reprobed with an anti-β-actin antibody.* ***b)*** *Cellular extracts (25 µg/well) were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with an anti-β-arrestin 1 antibody (see Table 1). Primary antibody binding was detected by incubation with an anti-rabbit secondary antibody linked to HRP. Membranes were stripped as described in the Materials and Methods section and after were reprobed with an anti-β-actin antibody.*

5.1.3 Effect of UVB irradiation on protein profile in the HaCaT cell line.

We wanted to determine the protein profile of HaCaT cells after UVB irradiation. To this aim, HaCaT cells were exposed to 30 mJ/cm^2 of UVB and were incubated for different times (3, 5, 9, 12 hours). In parallel, we used a non-irradiated control (Basal) and "sham-irradiated" samples after 3, 5, 9 and 12 hours of incubation (UV). Cellular extracts of HaCaT wt (25 µg) were resolved by SDS-PAGE electrophoresis and gel was stained with Coomassie Blue (see **Figure 13**).

Figure 13: ***Coomassie blue coloration of protein profile of HaCaT cells****.*



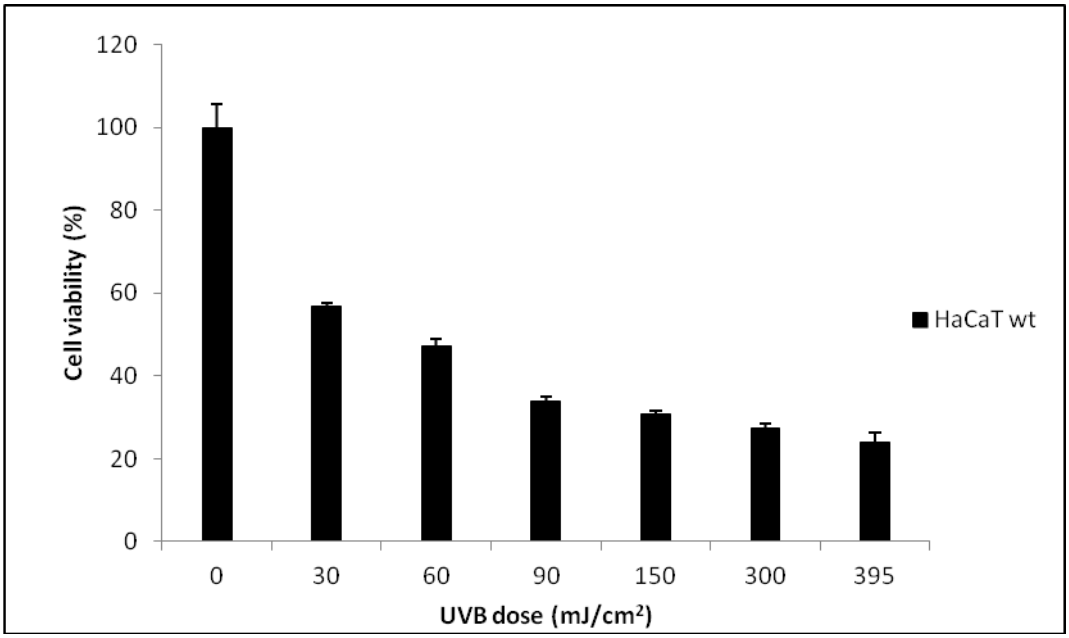
HaCaT cells were exposed to UVB radiation (30 mJ/cm²) and a part of the cells was kept in parallel (non-irradiated cells, Basal and condition «sham-irradiated», UV). After that, the cells were incubated for 3, 5, 9 and 12 hours. Cellular extracts (25 µg) were resolved by SDS-PAGE electrophoresis and the gel was stained with Coomassie blue as described in the Materials and Methods section.

The results show that irradiated and "sham-irradiated"- HaCaT cells have the same profile of proteins. Consequently, an irradiation of 30 mJ/cm^2 of UVB doesn't modulate the protein profile, revealed by Coomassie blue coloration, in the keratinocyte cell line HaCaT. Furthermore, we observed a band with a strong expression and with a molecular weight of about 60 kDa.

5.2 Effect of UVB irradiation on cytotoxicity on the HaCaT cell line

After the previous results we wanted to evaluate the cytotoxicity of UVB on the keratinocyte cell line HaCaT. We analyzed the viability of HaCaT cells after different doses of UVB (30, 60, 90, 150, 300 and 395 mJ/cm²). We used an MTT test which is a useful method for the measurement of *in vitro* cytotoxicity, viability and cell proliferation. Results are presented in **Figure 14**.

Figure 14: ***Viability of HaCaT cells irradiated with different doses of UVB***



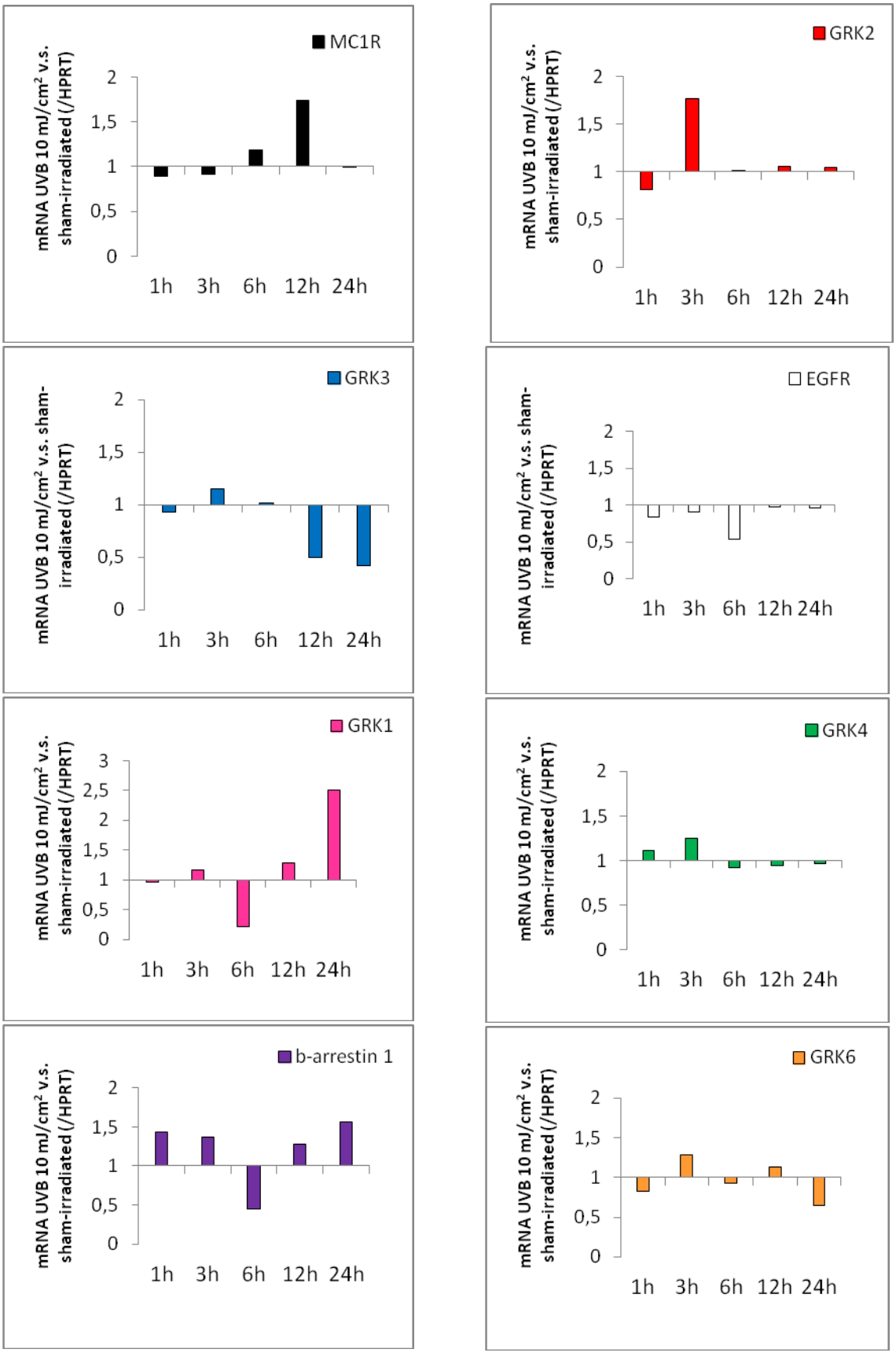
HaCaT cells were irradiated with different doses of UVB (30, 60, 90, 150, 300 and 395 mJ/cm^2). In parallel, a part of cells was not irradiated (non-irradiated control). The cells were seeded in a 96-microplate (100 000 cells/well) in DMEM without serum and were incubated for 18 hours at 37°C in 5% CO_2 in a humidified environment. MTT was added to the cells and was incubated until violet crystals appeared. The absorbance was measured at 570 nm and at 660 nm with a VICTOR microplate reader (PerkinElmer Life Sciences Inc.).

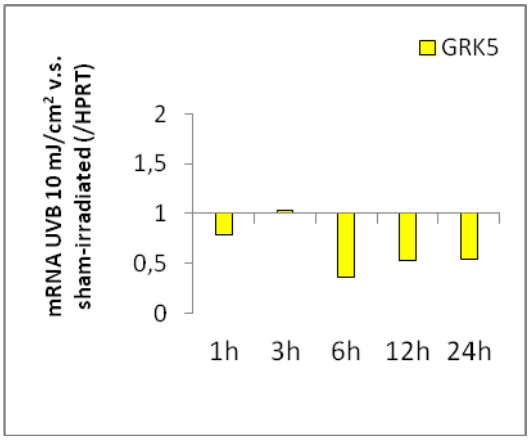
As we can see in **Figure 14**, UVB exposition had deleterious effects on the HaCaT cells' viability. We observed a linear decrease of viability when UVB irradiation increased. In fact, HaCaT cells have a mortality of (43,26 ± 0,73)%, (66,20 ± 1,06)% and (75,94 ± 2,18)% at 30, 90 and 395 mJ/cm^2 of UVB respectively.

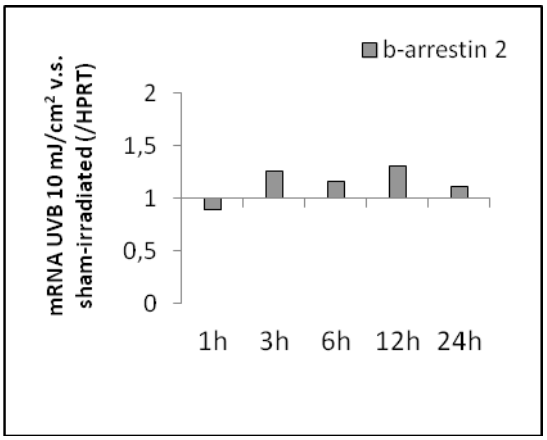
5.3 Genes expression in the HaCaT wt cells line irradiated by 10 mJ/cm2 of UVB

HaCaT wt cells were irradiated by 10 mJ/cm^2 of UVB. We used samples after different times of incubation – 1 hour, 3 hours, 6 hours, 12 hours and 24 hours. We tested MC1R, EGFR, GRK1 to 6 and β-arrestines 1 and 2 genes. Samples were normalized with hypoxanthine phosphoribosyltransferase 1 (HPRT). This whole experiment was carried out in duplicate, but only once at this time. Results are shown in the graphs in **Figure 15.**

Figure 15: ***Expression of genes the HaCaT wt cells line***







As we can see the expression of GRK5 is down regulated and β-arrestin 1 is up regulated in HaCaT cells after the UVB irradiation. We did not observe any consistent change in expression of other determined genes.

5.4 Development of a protocol of protein extraction with human epidermis *in vivo*

We compared three types of epidermis preparation with human skin as described in the Materials and Methods section (see **4.2.3.1** and **Figure 8**):

- Fresh epidermis (frozen skin)
- Frozen epidermis (fresh skin)
- Fresh epidermis (fresh skin)

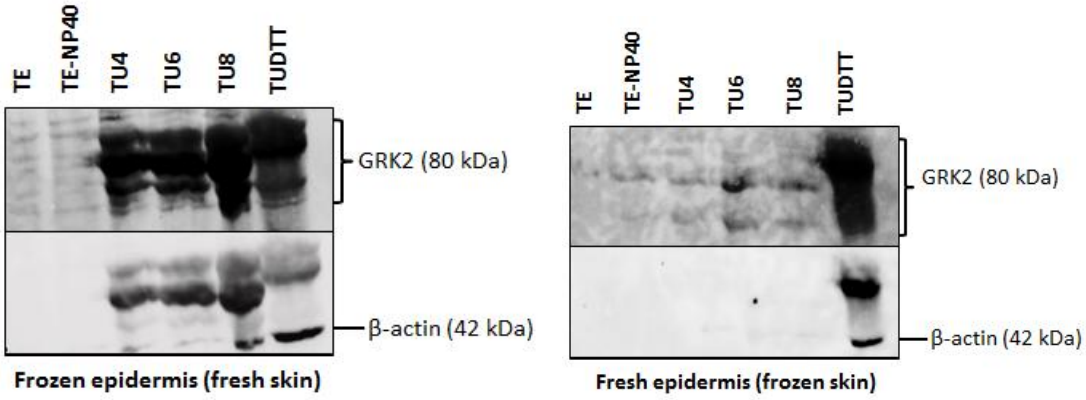
We used a sequential protein extraction protocol described by *Simon et al.(1977).* Epidermis was homogenized with a hypotonic buffer (TE) and with TE buffer containing NP-40 (TE-NP40). Obtained pellets were divided in three parts, which were extracted with TE buffer containing 4, 6 or 8 M of urea (TEU4, TEU6 and TEU8 respectively). After extraction, pellet obtained with TEU8 buffer was denaturated with a buffer containing DTT and glycerol (TUDTT).

The purpose of this study is to see if the freezing can be harmful for the preservation of the skin protein profile. Indeed, it is easier to work with frozen skin so according to needs and experiments. Furthermore, we want to finalize a method of protein extraction from *in vivo* human skin to realize biochemical studies with this type of material. Finally, we would like to work with biopsies of skin (4 mm^2) in the aim of comparing profiles between healthy and sick patients. This type of approach requires to be able to obtain enough proteins from little material.

5.4.1 Comparison of epidermis preparation revealed by Western-Blot

First, we compared two types of epidermis preparation with human skin: fresh epidermis (frozen skin) and frozen epidermis (fresh skin) and we compared the protein profile by Western-Blot with an anti-GRK2 antibody (**Figure 16**).

Figure 16: ***Expression of GRK2 and β-actin proteins in two types of epidermis preparation***



a) **b)**

a) Frozen epidermis (fresh skin). *We putted pieces of fresh skin in thermolysin (0,5 mg/ml in PBS) for 1,5-2 hours at 37°C in 5% CO_2 in humidified environment. After we separated epidermis of dermis, replaced epidermis in RNAlater®and safed them in liquid nitrogen. Before time using, epidermis was thawed in water bath (40°C, 5 min) and we made extraction.*

b) Fresh epidermis (frozen skin). *Pieces of skin were freezed in liquid nitrogen with RNAlater®. Before time using, we take of pieces of skin from liquid nitrogen and we thawed them in wather bath (40°C, 5 min). Then, we separated epidermis of skin after an incubation in thermolysin (0,5 mg/ml in PBS) for 1,5 – 2 hours at 37°C in 5% CO_2 in humidified environment and we make extraction.*

*Protein extracts (25 µg/well) were resolved by SDS-PAGE electrophoresis, were transferred to nitrocellulose membranes and were probed with an anti-GRK2 antibody (see section **4.1.3**). Primary antibody binding was detected by incubation with an anti-rabbit secondary antibody linked to HRP. Membranes were stripped as described in the Materials and Methods section and after were reprobed with an anti-β-actin antibody.*

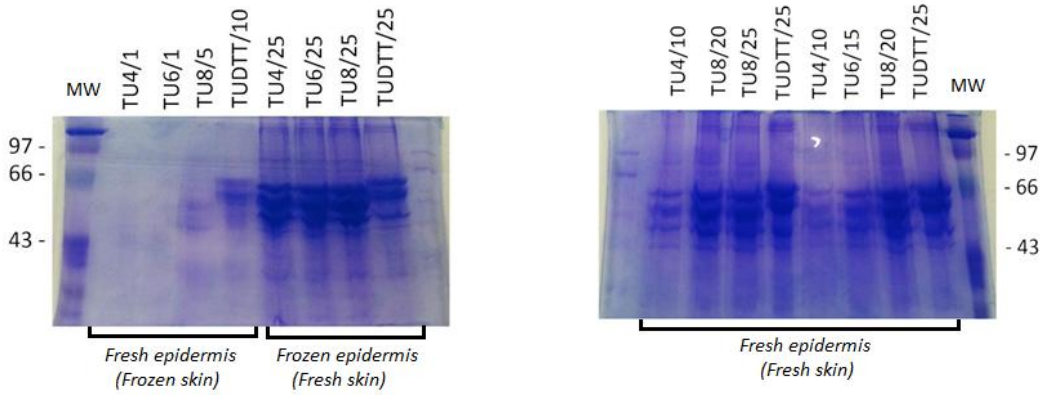
As we can see on **Figure 16a)**, for frozen epidermis (fresh skin), we observe GRK2 protein in the last 4 fractions (TU4, TU6, TU8 and TUDTT). GRK2 is extensively expressed in human epidermis. Nevertheless, it is difficult to conclude because the marking by the antibody reveals a thick band. In fact, we

observe the band of β-actin (42 kDa) in TU8 and TUDTT fractions but we observe again GRK2 binding. As we can see on **Figure 16b)**, for fresh epidermis (frozen skin), we observe only GRK2 protein in TUDTT fraction. Consequently, the protein profile in these two types of epidermis preparation is different.

5.4.2 Comparison of epidermis preparation revealed by Coomassie blue staining

Secondly, we compared the protein profile by Coomasie blue staining of three types of epidermis preparation with human skin : Fresh epidermis (frozen skin), Frozen epidermis (fresh skin) and Fresh epidermis (fresh skin). Protein extracts of human epidermis (1, 5, 10, 20 or 25 µg) were resolved by SDS-PAGE electrophoresis and gel was stained with Coomassie Blue (**Figure 17**).

Figure 17: ***Coomassie blue coloration of protein profile of human epidermis***.



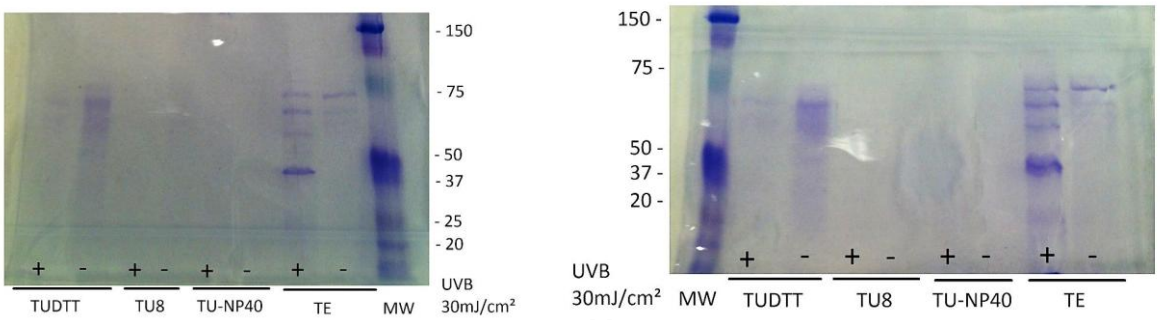
a) Fresh epidermis (frozen skin) *and* ***frozen epidermis (fresh skin).*** *Pieces of skin were freezed in liquid nitrogen with RNAlater®. Before time using, we take of pieces of skin from liquid nitrogen and we thawed them in wather bath (40°C, 5 min). Then, we separated epidermis of skin after an incubation in thermolysin (0,5 mg/ml in PBS) for 1,5 – 2 hours at 37°C in 5% CO_2 in humidified environment and we make extraction (fresh epidermis (frozen skin)).We putted pieces of fresh skin in thermolysin (0,5 mg/ml in PBS) for 1,5-2 hours at 37°C in 5% CO_2 in humidified environment. After we separated epidermis of dermis, replaced epidermis in RNAlater®and safed them in liquid nitrogen. Before time using, epidermis was thawed in water bath (40°C, 5 min) and we made extraction (frozen epidermis (fresh skin)).* ***b) Fresh epidermis (Fresh skin).*** *We had pieces of fresh skin and we placed them in thermolysin (0,5 mg/ml in PBS) for 1,5-2 hours at 37°C in 5% CO_2 in humidified environment. Then, we separated epidermis of dermis and made extraction. Protein extracts (1, 5, 10 15, 20 or 25 µg/well) were resolved by SDS-PAGE electrophoresis and gel was stained with Coomassie blue.*

As we can see on **Figure 17a)**, a concentration in protein between 1 and 10 µg is not sufficient to observe bands of any proteins on gel. It is thus necessary to work with concentrations in protein between 15 and 25 µg. The protein profile revealed by Coomassie blue is similar for frozen epidermis (fresh skin) and fresh epidermis (fresh skin) preparation (**Figure 17a) and 17b)**). Only the preparation from frozen skin seems to raise a problem because we extract a lower concentration of protein (**Figure 17A**).

5.5 Determination of protein profile induced by 30 mJ/cm^2 of UVB in human epidermis *in vivo*

Finally, we determined protein profile in human epidermis irradiated by 30 mJ/cm^2 of UVB. Skin was seeded (1 cm^2 per 100 mm Petri dishes) with PBS + 1% FBS and part of them are exposed to UVB radiation (30 mJ/cm²) and the second part was left on the table exposed (condition «sham-irradiated»). Skin was incubated during 2 hours in DMEM without serum (**Figure 2**). Thereafter, we separated epidermis of dermis and made extraction as described in the Materials and Methods section. Protein extracts of human epidermis (10 or 25 µg) were resolved by SDS-PAGE electrophoresis and gel was stained with Coomassie Blue (**Figure 18**).

Figure 18: ***Coomassie blue coloration of protein profile of UVB irradiated human epidermis***



a) **b)**

a) Coomassie blue staining with 10 µg of protein. *Skin was exposed to UVB radiation (30 mJ/cm²) and the second part was left on the table exposed (condition «sham-irradiated», UV). Skin was incubated during 2 hours in DMEM without serum. Thereafter, we separated epidermis of dermis and made extraction of protein. Protein extracts of human epidermis (10 µg) were resolved by SDS-PAGE electrophoresis and gel was stained with Coomassie Blue as described in the Materials and Methods section.*

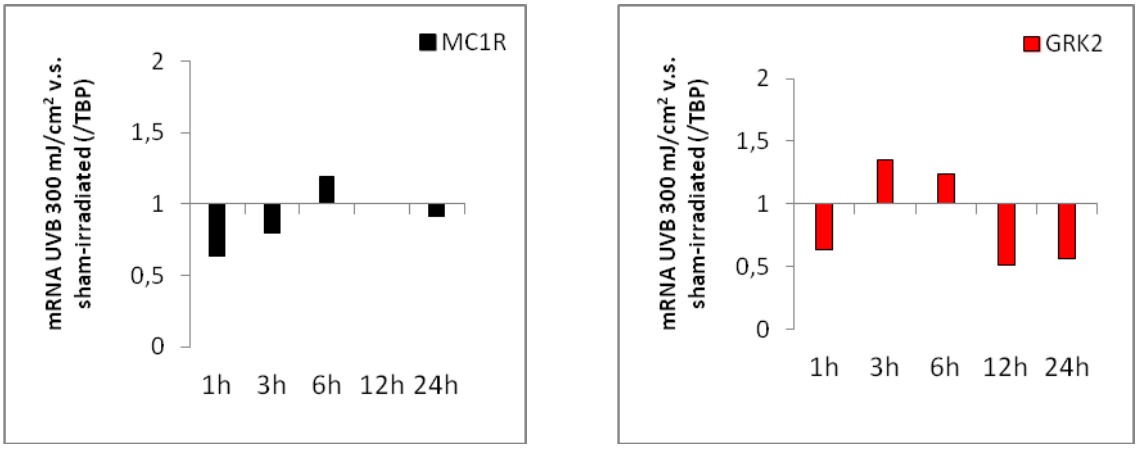
b) Coomassie blue staining with 25 µg of protein. *Skin was exposed to UVB radiation (30 mJ/cm²) and the second part was left on the table exposed (condition «sham-irradiated», UV). Skin was incubated during 2 hours in DMEM without serum. Thereafter, we separated epidermis of dermis and made extraction of protein. Protein extracts of human epidermis concentrated by TCA precipitation (25 µg) were resolved by SDS-PAGE electrophoresis and gel was stained with Coomassie Blue as described in the Materials and Methods section.*

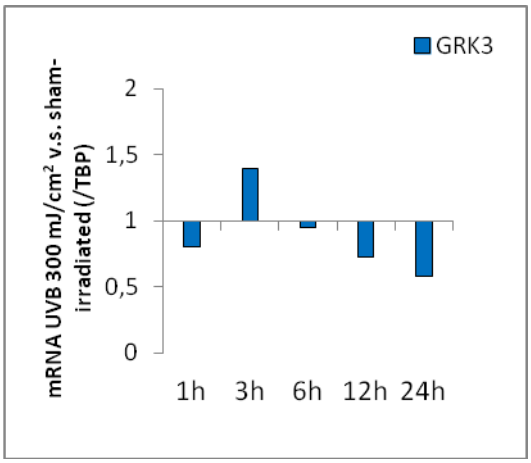
Results presented on **Figure 18** are very interesting because they show expression of an unknown protein with a molecular weight between 37 kDa and 50 kDa after UVB irradiation in the TE fraction. This unknown protein is not visible in ”sham-irradiated” samples. We observe these results at concentration of 10 µg (**Figure 18a**) and 25 µg of protein (**Figure 18b**).

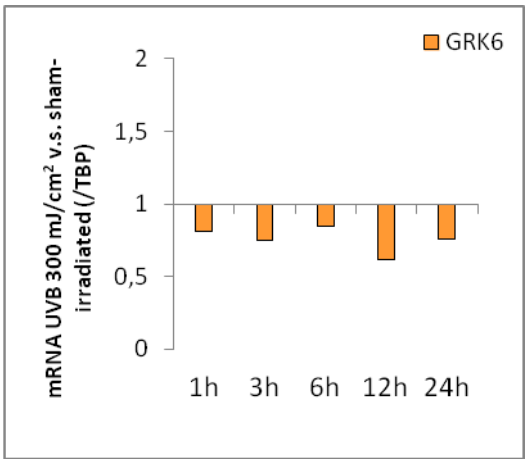
5.6 Genes expression in the human epidermis samples irradiated by 300mJ/cm^2 of UVB

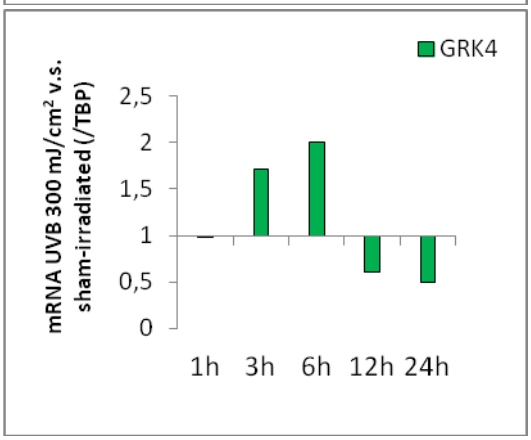
Human epidermis samples were irradiated by 300 mJ/cm^2 of UVB. We used samples of different times of incubation – 1hour, 3 hours, 6, hours, 12 hours and 24 hours. We tested MC1R, EGFR, GRK1 into 6 and β-arrestines 1 and 2 genes. Samples were normalized with TBP. Whole this experiment was made in duplicates but one time for the moment. Results are shown in the graphes on **Figure 19.**

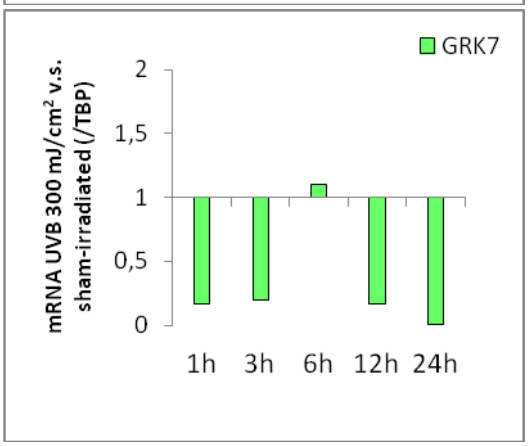
Figure 19: ***Expression of irradiated human epidermis skin samples***

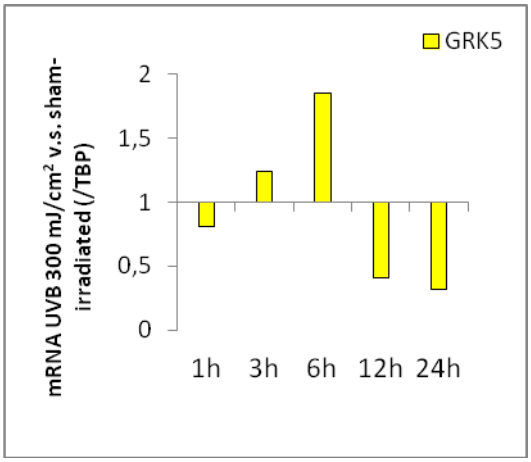


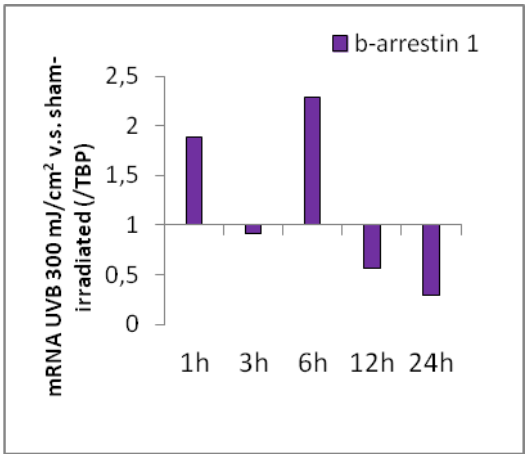

GRK3
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2
1,5
1
0,5
0
1h 3h 6h 12h 24h

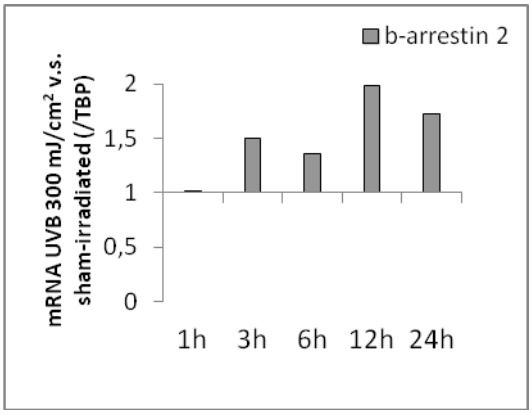

GRK6
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2
1,5
1
0,5
0
1h 3h 6h 12h 24h

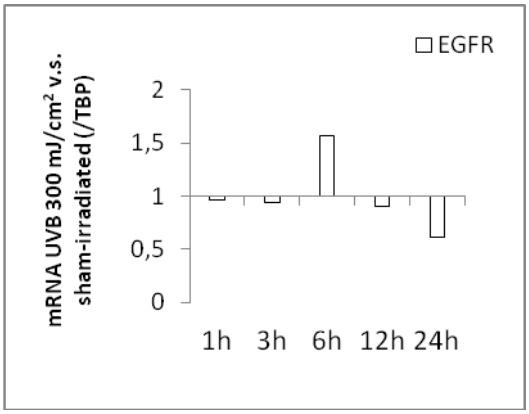

GRK4
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2,5
2
1,5
1
0,5
0
1h 3h 6h 12h 24h


GRK7
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2
1,5
1
0,5
0
1h 3h 6h 12h 24h


GRK5
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2
1,5
1
0,5
0
1h 3h 6h 12h 24h


b-arrestin 1
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2,5
2
1,5
1
0,5
0
1h 3h 6h 12h 24h


b-arrestin 2
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2
1,5
1
0,5
0
1h 3h 6h 12h 24h


EGFR
mRNA UVB 300 mJ/cm² v.s. sham-irradiated (/TBP)
2
1,5
1
0,5
0
1h 3h 6h 12h 24h

As we can see the expression of GRK7 is down regulated and β-arrestin 2 is up regulated in epidermis after UVB irradiation. We did not observe any consistent change in expression of other determined genes.

6 DISCUSSION

6.1 Modulation of GRKs and β-arrestin expression after the irradiation of UVB in HaCaT cell line

Our results demonstrated that, in the HaCaT cell line, UVB radiation increases expression of GRK2 protein between 5 and 9 hours after irradiation, at a later time period we observed a return to the basal level. We also found similar changes of GRK2 expression in mRNA level – the apparent increase of GRK2 expression was present 3 hours after UVB irradiation and at a later time expression was back at the basal level. We did not find agreement in changes in expression of any other GRK or β -arrestin determined on protein and mRNA level.

Regrettably we can not compare our result with any other studies because modulation of GRK2 in the HaCaT cell line after UVB irradiation in profusion has not been studied yet. Results by *Petronila et. al (2010)* suggest that altered GRK2 expression in specific tumour cells may affect cell migration and play a role in carcinogenesis, but not by comparing them after UVB irradiation.

It is important to say that we decided to change the dosage of UVB after the results from the MTT test. These results showed that with a UVB dosage of 30 mJ/cm^2 , the viability of cells was only about 50 %. We therefore decreased the dosage to 10 mJ/cm^2 UVB for qPCR measurement. For this reason it is very difficult to properly compare the changes in expression of the protein and mRNA level. Another problem is that these experiments are pilot studies, so the quantification was done only partly and a detailed conclusion could be expressed after finishing the whole project. Finally, since we decided to use a method with membrane stripping, it is very difficult to make quantitative comparisons between GRK2 and β-actin as the stripping procedure results in the removal of some of the sample protein from the membrane.

6.2 Modulation of GRKs and β-arrestin expression after the irradiation of UVB in epidermis

By contrast to HaCaT cells we did not observe changes in GRK2 expression in epidermis after UVB irradiation. We discovered that on the mRNA level there is an up-regulated expression of GRK7 and a down-regulated expression of β-arrestin 2. For now we can assess the results of epidermis only on the mRNA level with the qPCR method because it was not shown demonstrably with Western blot. The reason could be an inappropriate

dosage of the tested sample. Because we were at the beginning of testing, a different type of sample preparation was used.

6.3 Coomassie blue staining and modulation of unknown protein expression after the UVB irradiation

Results showed that UVB radiation does not importantly influence the change of protein profile in human epidermis. However by Coomassie blue staining we discovered a high expression of unknown protein after UVB irradiation. This protein has a molecular weight of about 37-50 kD. It is also interesting that this high expression was observed only in TE buffer. As *Dale et al., (1985)* suggests, keratins of 50 and 58 kD were present in the epidermis at all ages studied. Also results by *Krutmann et al (2008)* are proof that UVB radiation causes an increase in some types of keratin. Some of the tumour-associated keratin, such as keratin 6 and keratin 19, are significantly increased by UVB. Although we do not know which protein it may be, we can guess, that it might be keratin. Exact identification of this protein is beyond the scope of this work and is a subject for further research.

6.4 Epidermis samples preparation

We prepared 3 types of epidermis samples. Fresh epidermis (fresh skin), Frozen epidermis (fresh skin) and Fresh epidermis (fresh skin). Our results demonstrated that samples of Frozen epidermis (fresh skin) are quite usable but it would be better to decrease amount of protein. On the contrary, samples of Fresh epidermis (frozen skin) are inconvenient, because results from these demonstrated a low quantity of proteins. The reason may be the difficulty in keeping whole pieces of skin without prior epidermis separation. Finally, the samples of Fresh epidermis (fresh skin) are, according to our results, most suitable.

7 CONCLUSION

1) Modulation of GRKs and β-arrestin1 expression (on protein level) by UVB in HaCaT cells.
   - UVB irradiation increase expression of GRK2 between 5 and 9 hours after irradiation.
   - UVB irradiation does not modulate expression of GRK6, GRK1 and β-arrestin1 after UVB irradiation.
2) Determination of protein profile in the HaCaT cell line by the Coomassie blue staining method.
   - UVB irradiation does not modulate the protein profile in the HaCaT cell line.
3) Determination of cytotoxicity of HaCaT cells after UVB irradiation.
   - A dosage of 30mJ/cm^2 of UVB and higher induces a high cytotoxic effect on the HaCaT cell line.
4) Modulation of GRKs and β-arrestin1 expression (on mRNA level) by UVB in HaCaT cells.
   - UVB irradiation up-regulated expression of GRK5 and down-regulated β-arrestin 1.
5) Comparison of protocols for epidermis preparation,
   - Frozen epidermis (fresh skin) and Fresh epidermis (fresh skin) are both suitable for protein extraction (similar protein profile and sufficient amount of protein)
   - The model of Fresh epidermis (frozen skin) is not suitable (low concentration of protein in extraction).
6) Effect of UVB irradiation on the protein profile of human epidermis.
   - UVB irradiation induced expression of protein with a molecular weight between 37-50 kD.
7) Modulation of GRKs and β-arrestin1 expression (on mRNA level) by UVB in epidermis.
   - UVB irradiation up-regulated expression of GRK7 and down-regulated β-arrestin 2.

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ABSTRACT

GPCRs are types of receptors that are used for the transmission and communication between cells. GRKs and β-arrestins are specific proteins, which cause desensitization of GPCR and influence the transmission of signals to the final effecter cells. Recently, it was found out, that these specific proteins can also influence cell migration and play a role in carcinogenesis and UV induced cell death.

These types of GPCRs are also observed in skin. The skin introduces a protective shield against UV. UV light has strong genotoxic effects like DNA damage, mutation induction and, in the worst case, causes the growth of tumours.

The aim of our work was to determine the influence of UVB radiation on GRKs and β-arrestin gene expression in HaCaT cell lines in vitro and in human epidermis in vivo. We also made efforts to compare different types of preparation of epidermis samples.

For this work we used SDS-PAGE, Western blot, Coomassie blue staining and qPCR. We also used the MTT test for determination of viability on HaCaT cells line.

On protein level our results showed increasing expression of GRK2 in HaCaT cell lines after UVB irradiation. To the contrary on the mRNA level we investigated the up-regulation of expression of GRK5 and down-regulation of β-arrestin 1.

On the mRNA level, results with epidermis after UVB irradiation showed an up-regulation expression of GRK7 and a down-regulation of β-arrestin 2. Our results with epidermis samples preparation demonstrated that samples with Fresh epidermis (fresh skin) are the most suitable.

ABSTRAKT

Receptory spřažené s G proteinem (GPCRs) jsou typy receptorů, které slouží k přenosu signálu a ke komunikaci mezi buňkami. Kinázy receptorů spřažené s G proteinem (GRKs) a arrestiny jsou specifické typy proteinů, které jsou schopny desenzitizace těchto GPCRs a zároveň tedy ovlivňují přenos signálu k cílovým buňkám a také délku trvání tohoto signálu. Nedávno bylo také objeveno, že tyto specifické proteiny mohou ovlivňovat migraci buněk, podílí se na karcinogenezi a na UV zářením indukované smrti buněk.

Typ GPCRs receptorů se nachází také v kůži, která představuje ochranný štít před UV zářením. UV vykazuje silný genotoxický efekt, může způsobovat poškození a mutace DNA, a v nejhorším případě, může také vést k tvorbě nádorů.

Cílem naší práce bylo zjistit, jaký vliv má UV záření na genovou expresi GRKs a beta arrestinu v buněčné linii HaCaT *in vitro* a v lidské epidermis *in vivo*. Snažili jsme se také o porovnání různých typů protokolů pro manipulaci s kůží a pro přípravu testovaných vzorků.

Pro tuto práci jsme použili metody jako SDS-PAGE a Western blot, barvení pomocí barviva Coomassie blue pro zjištění proteinového profilu a kvantitativní typ PCR. Pro testování viability buněčné linie HaCaT jsme využili MTT test.

Naše výsledky ukazují, že na proteinové úrovni buněčné linie HaCaT po ozáření UVB zářením, došlo k vzestupu exprese GRK2. Naproti tomu na úrovni mRNA jsme pomocí PCR odhalili zvýšenou regulaci exprese GRK5 a sníženou regulaci exprese β-arresinu 1.

Vzorky kůže byly porovnatelné pouze na úrovni mRNA a tady jsme pozorovali zvýšenou expresi GRK7 a β-arrestinu 2 po ozáření UVB.

Co se protokolů pro přípravu vzorků epidermis týče, tak jsme dospěli k závěru, že nejvhodnějšími vzorky jsou vzorky čerstvé epidermis z čerstvé kůže.