FINAL REPORT

OTKA 105872 (2012.09.01-2016.10.31.)

Principal investigator: Eva Remenyik

INTRODUCTION

Ultraviolet (UV) radiation, especially UVB (290-320nm) is the most important environmental stressor for the human skin**. It's acute high dose induces** erythema and epidermal damage (sunburn), chronic sun exposure responsible for photoaging and skin tumors[16] with high and increasing healthcare cost[20]. UVB irradiation causes the formation of two major photolesions in DNA, namely the **cyclobutane pyrimidine dimers (CPDs**) and the 6-4 pyrimidine-pyrimidine photoproducts (6-4PPs). CPDs are the most important photolesions based on their high abundance and slow repair, may cause cytotoxic and mutagenic effects on the cells contributing significantly to acute **sunburn** and also **UVB-induced carcinogenesis**[22, 26, 27]. These photoproducts are normally removed from the genome by the nucleotide excision repair (NER)[19]. Apart from NER mechanism, photoreactivation is another powerful repair system for removal of UVB induced DNA lesions which is carried out by photolyases**. Photolyase** is an enzyme that specifically recognize and repair CPDs or 6-4PPs by converting UV lesions back to the original state using visible light energy (photoreactivation) and has **photoprotective effect**. However, photolyases are present in many organism (ranging from bacteria to marsupials), these enzymes are absent in placental mammals[18]. There are some studies on reversing UVB effect by introducing liposome containing photolyase enzyme on cell culture[24] and also in clinical settings[23] but scientific evidences of the nuclear localization of the protein are missing. Study the effect of photolyase expression after **gene transfection** in mammalian cells are rare in the literature[2].

Photolyase expressing cells are an excellent and essential model to study the detailed mechanisms of UVB effects on cells and tissues. These models allow distinguishing different cellular mechanisms initiated by UVB. Using CPD photolyase transfected cells, CPD driven processes after UVB irradiation can be separated from other suspected effects of UVB (cell membrane, oxidative, direct protein etc.). DNA based gene delivery has major disadvantages mainly the intercalation of gene and/or viral vectors into the host genomic DNA. There are several methods to avoid this unwanted consequence of DNA based gene therapy, among them the most promising in clinical point of view is the mRNA based gene transfection[15]. We have had an opportunity; financed by previous research grant; to collaborate with Katalin Kariko's research group who has been working on **mRNA based transfection.** They found that mRNAs containing a modified nucleoside, especially pseudo-uridine, increases translational capacity, stability of mRNA and eliminates immune responses[1, 13, 14, 25].

In our previous work, we identified that i**n vitro synthesized, pseudo-uridine -modified CPD specific photolyase mRNA** (a photolyase gene derived from the marsupial rat kangaroo, Potorous tridactylus) complexed with lipofectamine-LTX transfection reagent could successfully introduced into human keratinocytes. The encoded photolyase was detectable already one hour after transfection and was maintained for 3 days. Translated photolyase **was functionally active**; almost all CPDs were repaired in photolyase mRNA transfected cells exposed to photoreactivating light compared to those kept in dark. Photoreactivation also significantly reduced the antiproliferative effect of UVB irradiation in human keratinocytes, it had photoprotective effect. Our data showed that enhanced repair of CPDs by photolyase mRNA significantly reduced the UVB induced IL-6 production in human keratinocytes, suggesting that the release of IL-6 after UVB irradiation is mediated by DNA damage, and CPDs are the major components in this process.

Besides DNA damage, chronic reduction in energy production after UVB can also be observed which currently attributed to the cumulative damage to mitochondria. Therefore, appropriate metabolic changes also play important role in mediating the UVB-induced stress. Poly (ADP-ribose) polymerases can be a part of this response, however their role in the UVB-driven changes are quite elusive. The most characterized part of the family is the PARP-1 which take part in several cellular processes, including cell division, cell death, DNA repair, transcription, cell division, inflammation and metabolism, well-reviewed by Bai P[3]. PARP-1 activation takes place after UVB and there is direct link (NAD^+, ATP depletion, Sirtuin inactivation) between the deteriorating functionality of mitochondria and PARP-1 overactivation. Sirtuins are class III-deacetylases and like PARP-1, they use NAD^+ in their enzymatic reaction. However, the activation of PARP-1 and SIRT-1 are mutually exclusive[8]. PARPs also regulates inflammation[4]. Nowadays a great number of PARP inhibitors are in clinical trials used in patients with different type of malignant disorders. However, the role of PARP-1 in regulating the UVB-induced cellular responses are hardly known.

(Péter Bai, László Virág: Role of poly(ADP-ribose) polymerases in the regulation of inflammatory processes, ***FEBS Letters 586, 3771–3777, 2012 IF:3.986***

Carles Cantó, Anthony A. Sauve, Peter Bai: Crosstalk between poly(ADP-ribose) polymerase and sirtuin enzymes, ***Molecular Aspects of Medicine, 34, 6, 1168–1201, 2013 IF:4.739)***

UV radiation is an important etiological factor not only in keratinocyte skin cancers, but also in malignant tumors derived from melanocytes. Melanoma malignum, with high metastatic potential is the most aggressive skin tumor. Besides tumor thickness, exulceration and mitotic rate other prognostic factors would be important in primary melanomas to predict the outcome and establish treatment strategies.

Zinc homeostasis of the cell is essential for regulation of proliferation, apoptosis and regeneration; several zinc dependent enzymes, transcription factors are involved in these processes[12]. We have already started to examine the significance of **metallothionein** (MT), a Zn binding protein, in the prognosis of cutaneous malignant melanoma (CMM) using clinical and histopathological data. With the help of this grant we were able to finish this work and published, that the expression level of **metallothionein in primary melanomas correlates with the tumor promoting host immune response[9]**

(Emri E, Egervári K, Várvölgyi T, Rózsa D, Mikó E, Dezső B, Veres I, Méhes G, Emri G, Remenyik, É.: *Correlation among metallothionein expression, intratumoural macrophage infiltration and the risk of metastasis in human cutaneous malignant melanoma.,* **J. Eur. Acad. Dermatol. Venereol 27:3: 320-327, 2013 IF 2,98)**

The role of MT in skin is hypothesized to be protective against UVB radiation, under physiological conditions, however, it directs cells toward malignancy under pathological conditions[17]. Nevertheless the effect of zinc on UV has not yet been fully explained.

METHODS

Cell culture of HaCaT cells, melanocytes, primary keratinocytes were performed. UVB irradiation using Philips TL12 light source. ELISA was used for checking the efficiency of repair mechanism, which can detect the amount of CPDs in DNA. TILDA and Microarray technology were used to generate global gene expression profiles after zinc treatment and also UVB irradiation and after transient transfection (using lipofectamine) of CPD-specific photolyase mRNA into human keratinocytes. Ingenuity Pathway analysis (IPA), a web based application, was

used for identification of significantly overrepresented biological pathways, mechanisms and interactions (networks) between differentially regulated genes in our datasets. Validations were done by qRT-PCR. Results for genes aberrantly expressed after UVB and CPD repair were confirmed at the protein level, using western blot analysis. Mitochondrial membrane potential, OCR/ECAR determination. ELISA, confocal microscopy, flow cytometry, and cell viability assays, immunocyto-, histochemistry and electron microscopy were also done. Applied antibodies: anti CPD, p53, Ki67, CCNE1 GADD45, Panoramic Viewer software.

Our Aims were:

to investigate the events after UVB-induced DNA damage to better understand the pathomechanism of UVB irradiation.

1. Detection of new CPD driven pathways and novel possible regulations

2. Determination of CPD dependence or independence of known UVB induced cellular processes.

3. Development of *in vivo* mRNA delivery system(s) to skin.

RESULTS:

1.1.With the help of this grant we were able to finish (repeat and completed some experiments, write the final version) our previous results according to the requirements of the referees of the journal and finally published the **successful transfection of CPD photolyze *in vitro* synthesized mRNA into human cultured keratinocyte.**[6]

*(Boros G, Mikó E, Muramatsu H, Weissman D, Emri E, Rózsa D, Nagy G, Juhász A, Juhász I, Van der Horst G, Horkay I, Remenyik É, Karikó K, Emri G: Transfection of pseudouridine-modifien mRNA encoring CD-photolyase leads to repair of DNA damage in human keratinocytes: A new approach with future therapeutic potential**, J Photochem Photobiol B. 129:93-99., 2013 IF:3,11)***

1.2 **Description of CPD driven and independent gene-expression changes of human keratinocytes after UVB irradiation**

With our previous work we have established a model system in which 90% of UVB-induced CPDs could be removed after exposing to photoreactivating light by photolyase protein expressed from exogenous mRNA. Using this model and oligonucleotide microarray, we have determined gene expression changes in human keratinocytes subjected to 20 mJ/cm2 UVB at 12 h post-transfection followed by photoreactivation or incubation in the dark. This model system allowed us to investigate not only the removal of UVB-induced DNA lesions, but, more importantly, this platform can be applied to identify and distinguish CPD dependent and independent molecular and cellular processes. Interestingly, more than half of UVB-mediated genes were CPD dependent. With Ingenuity Pathway Analysis (IPA), a web-based software application, we determined that a majority of CPD-dependent gene expression changes at 6 and 24 h after UVB exposure is linked to decreased cell viability and apoptosis induction. Interestingly, gene expression changes that were independent of CPDs seemed to influence the cell viability in the opposite direction at 24 h after UVB irradiation. These data demonstrated that CPDs are principal contributors to the UVB-induced decrease in cell viability. These differentially expressed genes were associated primarily with cell cycle, cell growth, cell differentiation which regulated mostly through the TGF-beta and *Wnt* cell signaling pathways. The changes observed in the microarray experiments were also validated

by RT-qPCR in case of the highest differentially regulated genes: ADAMTS1, CDKN1, ETS2, MARCKSL1, GSG2, GADD45A. The results of qRT-PCR confirmed microarray data. In protein level we also confirmed an increase in the GADD45A protein after UVB

In response to effects of CPD, we identified a few new target genes. 10 selected CPD-related genes real time quantitative RT-PCR (RT-qPCR) analyses also were done. The result of qRT-PCR demonstrated the significant upregulation of mRNA expression for ATF3, CCNE1, CDKN2B, EGR1, ID2, PTGS2, SNAI1, SNAI2 and the downregulation of RUNX1 and SMYD3 in response to UVB at 6 or 24 h after the exposure in CPD-PL Ψ-mRNA transfected cells that left in the dark. It is remarkably that altered expression for each of these genes was returned to normal range due to photoreactivation of keratinocytes transfected with CPD-PL Ψ-mRNA. These results confirmed that expression pattern of ten selected genes were CPD-dependent in good agreement with those derived from microarray analyses and highlight some possible new molecular targets in UVB-induced cellular responses.

We also validated of the results from microarray analysis at protein levels. We selected two CPD-dependent cell cycle-regulatory genes (CCNE1 and CDKN2B) that were previously less characterized related to their involvement in UVB-induced stress response. We have found that the expression of cyclin E1 and p15INK4b proteins were significantly increased at 24 h after UVB irradiation, but this increase was prevented by active CPD-photolyase.

Using LSC method we would like to investigate the contribution of CPD for activation of apoptotic signaling and cell cycle arrest in a time- and dose-dependent experiment in HaCaT cells containing active or inactive CPD-photolyase. In this context, we showed that the CPD-photolyase mediated repair of UVB-induced CPDs prevented increased expression of two cell-cycle regulatory genes, CDKN2B (p15INK4b) and CCNE1, mRNA and protein. Despite the apparently opposite function of CDKN2B and CCNE1, we found that UVB irradiation triggered overexpression of both genes in human keratinocytes. However, the effect of a cell cycle regulatory gene on cell cycle progression is highly dependent on cellular levels of other regulatory molecules and their posttranslational state. To investigate the signaling pathways involved in modulation of cyclin E1 and p15INK4b expression upon UVB exposure, HaCaT cells were exposed to specific inhibitors of JNK, p38 MAPK or AKT followed by UVB irradiation. We determined the expression of cyclin E1 and p15INK4b proteins by western blot analysis. While suppression of p38 MAPK or AKT had no effect on UVB-induced increase in the levels of these proteins, we found that the treatment of cells with the JNK inhibitor abolished the induction of cyclin E1 and p15INK4b protein expression at 24 h after UVB. Our findings suggested the involvement of **JNK signaling pathway in CPD-dependent cellular stress response**s upon UVB exposure. The results of the photolyase experiments were published[5, 7].

(Gábor Boros, Edit Miko, Hiromi Muramatsu, Drew Weissman, Eszter Emri, Gijsbertus van der Horst, Andrea Szegedi, Irén Horkay, Gabriella Emri, Katalin Karikó, Éva Remenyik: *Identification of cyclobutane pyrimidine dimer-responsive genes using UVB-irradiated human keratinocytes transfected with in vitro-synthesized photolyase mRNA*, **PLoS One 29;10(6):** e0131141. **2015 IF:3.534**

Gábor Boros, Katalin Karikó, Hiromi Muramatsu, Edit Miko, Eszter Emri, Csaba Hegedűs, Gabriella Emri, Éva Remenyik: *Transfection of human keratinocytes with nucleoside-modified mRNA encoding CPD-photolyase to repair DNA damage*, **Methods in Molecular Biology 1428:219-28. 2016 IF:1.29)**

1.3. **Immunohistochemistry in human precancers and skin cancers**

We also carried out immunohistochemistry on human samples, to investigate the role of the CPD-dependent CCNE1 in non-melanoma skin cancers, including basal cell carcinoma, squamous cell carcinoma and keratoacanthoma and also solar keratosis. To determine the expression of **CCNE1 o**n sun-exposed areas of the skin immunohistochemistry was used. We have found that the expression of CCNE1 was increased in only patients suffered from squamous cell carcinoma (n = 2).

In a photocarcinogenic point of view we extended our research to more close to the dermatological practice. Actinic keratosis are the clinical signs of the precancerous UV induced lesions, so consequences of the CPD formations. We have started to investigate whether Er:YAG ablative fractional pretreatment of actinic keratosis can increase the therapeutic efficacy and photo-rejuvenation effect of conventional **photodynamic therapy**. In a pilot study, we detected the **p53 positive cells** by immunohistochemical staining and analyzed their number with Pannoramic Viewer software. Both treatment modalities reduced the number of p53 positive cells 3 month after the therapy, but only the PDT mediated decrease of these cells was statistically significant.

(**Posters**: Therapeutic efficacy and photorejuvenation effect of Er:YAG laser assisted photodynamic therapy compared to conventional photodynamic therapy in patients with multiple actinickeratosis. Emese Gellén, Eszter Janka, Gabriella Emri, Éva Remenyik, 6th 5CC Cannes, 2015,

Therapeutic efficacy and photorejuvenation effect of Er:YAG laser assisted drug delivery compared to conventional photodynamic therapy in patients with multiple actinic keratosis. Emese Gellen, Eszter Janka, Barbara Barta, Gabriella Emri, Eva Remenyik 25th EADV, Vienna, 2016)

2. 1. Since UV has crucial role in the formation of melanoma, we aimed to investigate the relationship between **zinc and the effect of UVB.**

Zn –TILDA
HaCaT cells were treated with ZnCl2 for 4 or 24 h, and relative gene expression was measured. In a parallel experiment, cells were pre-treated with ZnCl2 for 24 h and then UVB-irradiated. 6 h after exposure, total RNA was isolated and cDNA was synthesized. We designed Custom TaqMan Array Microfluidic Cards (384-well); we selected 91 genes because their roles have been reported in connection with a UVB response and zinc. The selected genes are involved in cell cycle progression, inflammation, apoptosis, DNA repair, and antioxidant defense. Genes connected with zinc homeostasis and some with identified MRE in the promoter region were also added. Data were analyzed using SDS 2.1 software. The ACTB, GAPDH, SDHA, and PGK1 were used for normalization. Relative gene expression values were calculated using the 2-ddCt method. As results, we confirmed data derived from microarray analysis of UVB irradiated and CPD-photolyase mRNA transfected (CPD repaired) keratinocytes. 39 differentially expressed genes were identified in cells only UVB-irradiated 6 h after exposure compared to non-irradiated, control cells. Twelve genes were expressed more than 2-fold higher in UVB-irradiated cells than in control among them, TNF, PTGS2, and IL-8 showed the highest overexpression (> 10-fold). We identified 27 genes that were down-regulated in UVB-irradiated cells compared to control; MT1E was the most repressed (0.12-fold). Furthermore, MTF1 significantly decreased compared to control (0.32-fold). We observed a modest modulation in gene expression comparing zinc pre-treated, UVB-irradiated cells to those only UVB-irradiated. Zinc pre-exposure mainly reduced the UVB-induced HIPK2 mRNA suppression and caused little modification of POLB and RAD51 mRNA levels; relative gene expression level was repressed followed by UVB irradiation (< 0.75-fold) and was altered to remain unchanged (0.76-1.5 fold change) or did

not change significantly from control after zinc pre-treatment. In addition, expression of zinc homeostatic genes (MT1F, MT1X, MT2A, and SLC30A1) was up-regulated followed by the zinc and UVB combination treatment.

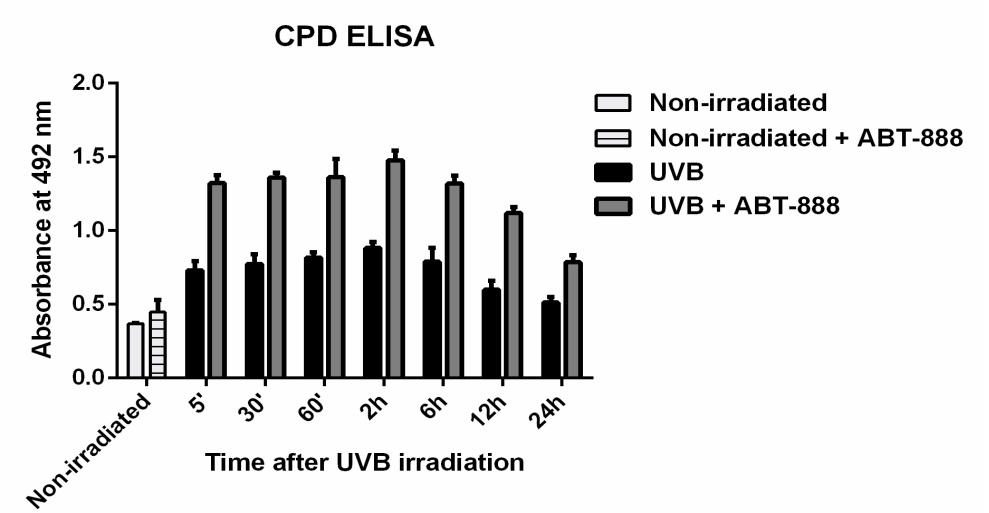
Effect of zinc and UVB co-exposure on metallothionein protein
We were interested then whether Zn (II) pre-treatment whether has an effect on UVB-induced reactive oxygen species production. Consequently, we characterized UVB-induced O2•− generation and cyclobutane pyrimidine dimer (CPD) formation after 24-h Zn (II) pre-treatment. We observed an increase in O2•− generation, with a significant plateau 10 h post-UVB irradiation compared to the control (1, 4, 10, and 24 h after UVB exposure), and we detected a time-dependent decrease in the amount of CPDs after UVB irradiation (0, 1, 3, 6, and 24 h after UVB exposure). Zn (II) pre-treatment changed both processes that were induced by UVB. Our results showed that significantly less CPDs were detected 3 h after UVB irradiation in cells pre-treated with Zn (II) for 24 h compared to cells exposed only to UVB. However, at 10 h post-UVB exposure, significant enhancement of superoxide generation was observed when comparing Zn (II) pre-treated, UVB-irradiated cells to cells only treated with UVB irradiation.
To investigate the effect of reduced levels of CPDs but elevated O2•− levels after Zn (II) pre-treatment, apoptosis and cell viability were measured. We found that the cell survival did not change between Zn (II)-treated and control cells 24 h after UVB irradiation. Regarding the ratio of apoptotic cells, the proportion of viable cells during Zn (II) exposure was similar as observed in Zn (II) pre-treated, UVB irradiated cells. On the other hand, Zn (II) pre-treatment significantly decreased the proportion of early apoptotic cells (Annexin V+PI-) and significantly increased the population of late apoptotic plus necrotic cells (Annexin V+PI+ and Annexin V-PI+). As we found an effect of Zn (II) pre-exposure on UVB-induced CPD formation we investigated the cellular distribution of MTI/II, whether there is any nuclear translocation of this protein. MTs were present mainly in the nucleus of the untreated cells and we found that Zn (II) induced immunostaining of MTs in the cytosol. UVB treatment changed the picture of Zn (II) pre-exposure, partial translocation of MTs to the nucleus was observed 3 h after UVB irradiation.
Our results, that we observed changes in ROS, suggest molecular effects of Zn (II) interactions with cysteinyl thiols, which alters protein functionality and thereby their reactivity and participation in redox reactions. It can affect signal transduction pathways, which further may influence the behavior of the cell upon stress conditions. Our observations affect our thinking about the pathogenesis of skin disorders and pay attention to further examine the role of the zinc homeostasis in skin, which findings were published[10].

(Eszter Emri, Edit Miko, Péter Bai, Gábor Boros, Georgina Nagy, Dávid Rózsa, Tamás Juhász, Csaba Hegedűs, Irén Horkay, Éva Remenyik, Gabriella Emri: *Effects of non-toxic zinc exposure on human epidermal keratinocytes*, **Metallomics, 2015 Mar;7(3):499-507. doi: 10.1039/c4mt00287c., 2015 IF: 3.585)**

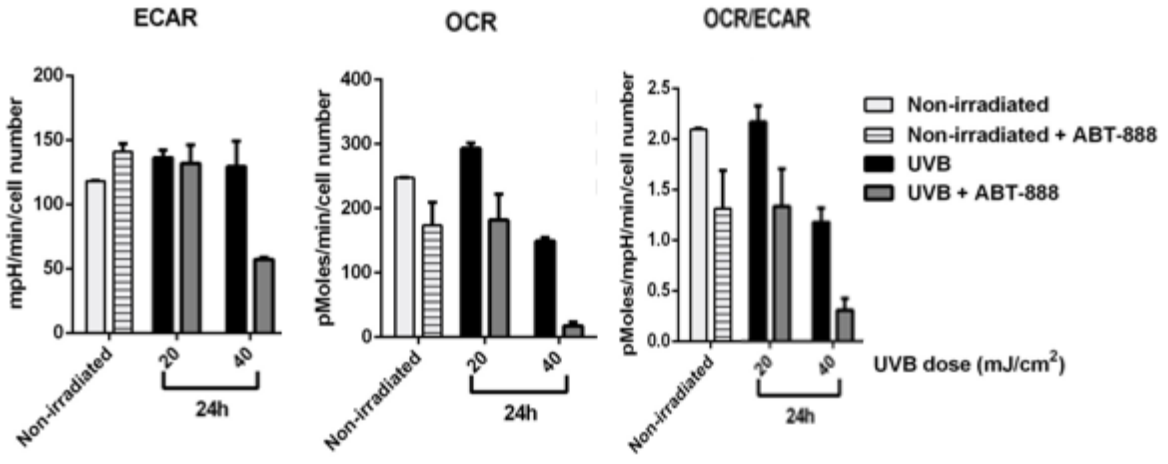
2.2 **PARP-1 and mitochondria**

Since mitochondrial DNA damage is sensitive biomarker for UVB irradiation and there is an established connection between PARP-1 activation and mitochondrial damage, we examined how PARP-1 inhibition (Veliparib/ABT-888 treatment) affects the metabolic capacity of cells after UVB.

PARP-1 inhibition reduced cell viability at both 20 mJ/cm^2 and 40 mJ/cm^2 at 24 hours after UVB irradiation. At lower dose both the Annexin V-positive and PI-positive cells were increased, at 40 mJ/cm^2 dose, the number of necrotic cells were decreased, the cell death pathway shifts toward apoptosis. PARP-1 inhibition also sensitized keratinocytes to UVB. It resulted in the elevation of CPDs immediately after UVB and induced the retention of CPDs which corroborates its role in DNA repair including NER.



Regarding the energy homeostasis of cells, UVB slightly reduced NAD and ATP content of keratinocytes. Veliparib treatment in the non-irradiated samples, robustly increased the bioavailability of NAD, and slightly increased the NAD/NADH ratio after UVB. Similarly, ATP content was also raised under PARP inhibition. UVB also affected the metabolic phenotype of cells. The ECAR referring to glycolysis remained unchanged, the OCR reflecting the rate of oxidative phosphorylation diminished. Interestingly, PARP-1 inhibition could not provide any protective effect against the plummet in the OCR/ECAR ratio, it even further decreased mitochondrial respiration.

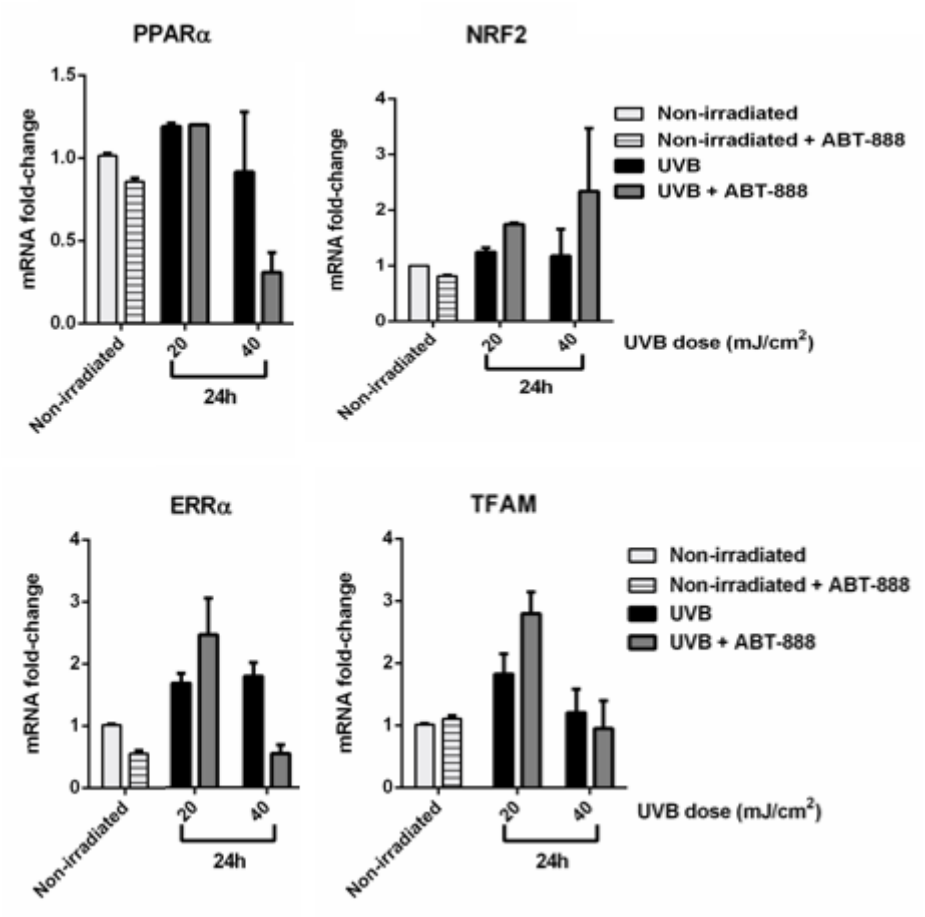


Together these data suggest that the elevated ATP level stems from glycolysis rather than oxidative phosphorylation. Similar mRNA expression changes were detected in the genes which build up mitochondrial electron transport. PARP-1 inhibition decreased the expression of IDH2, Ndufb5, ATP5g1 and increased Ndufa2, COX4, CYCS expression. As a mitochondrial dysfunctional parameter, UVB triggered mitochondrial membrane

hyperpolarization and reactive oxygen species (ROS) generation. This dysfunctionality is indeed the consequence of PARP-1 activation, since veliparib treatment, attenuated both mitochondrial hyperpolarization and ROS formation. NRF2 and SOD2 can be emphasized in mediating the beneficial changes which are both part of the antioxidant response, and were upregulated by PARP inhibition.

Regarding gene expression changes, UVB time-and dose-dependently downregulated the SIRT-1/3/4, where PARP-1 inhibition provide protection at mild UVB dose. The expression of sirtuin-regulated transcription factors (PGC1a/b, FOXO1/3) involved in fatty acid and carbohydrate oxidation were increased at 24 hours and veliparib treatment further augmented the mRNA level of these genes. Veliparib treatment also caused changes in p-AKT, GSK3a/b, AMPK, p-AMPK, p-ACC, SIRT-1 protein level all of them involved in metabolism, but we have to be careful in the interpretation of these results and require further experiments. We can conclude that PARP inhibition activates energy-producing pathways at mild UVB dose, and the activation of sirtuins prepares the cells to the altered energy demand at stress conditions.

The PGC1a coactivators (PPARa, NRF2, ERRa, TFAM) also showed similar gene expression patterns like SIRT-regulated transcription factors.



Together these genes regulates pathways that ultimately lead to mitochondrial biogenesis. Electron microscopy experiments shows that UVB dose-independently ruined the mitochondrial inner membrane and the classical elongated mitochondrial morphology was switched to rounded dispersed structures. UVB also increased mitochondria number without increasing mitochondrial mass. In contrast, PARP inhibition in some extent showed protective

effect on mitochondrial morphology. The inner membrane remained intact, and the mitochondrial morphology remained elongated like in the non-irradiated control group. Moreover, veliparib treatment further increased mitochondria number with elevated mitochondrial mass. Together these data suggest that, UVB induces structural deterioration, mitochondrial fragmentation, and PARP inhibition induces mitochondrial biogenesis.
Taking these results into consideration, mitochondria and metabolic switches have great deal of importance in mediating adaptation response following acute stress. PARP-1 inhibition at mild UVB dose provide almost complete protection against the UVB-induced mitochondrial dysfunction, and induced mitochondrial biogenesis by activation SIRTs and PGC1a-coactivated nuclear receptors and transcription factors.

(Some selected posters:

Csaba Hegedűs, Gábor Boros, Eszter Emri, Edit Mikó, Tamás Fodor, Katalin Karikó, Gréta Nikoletta Kis, Eszter Anna Janka, Gabriella Emri, Miklós Antal, Péter Bai, Éva Remenyik The role of Poly (ADP-ribose) polymerase-1 in the UVB-driven metabolic changes on HaCaT keratinocytes ESDR 2016, Munich. Journal of Investigative Dermatology (JID), Vol. 136, Issue 9, S252

Csaba Hegedűs, Gábor Boros, Eszter Emri, Edit Mikó, Katalin Karikó,Gabriella Emri, Péter Bai, Éva Remenyik The implication of mitochondria in the UVB-driven pathways. ESDR 2014, Copenhagen. Journal of Investigative Dermatology, Vol. 134, S83–S89)

LESS DETAILED EXAMINED ORIGINAL AIMS:

Evaluation of chromatin structure.

UVB irradiation changes the overall supercoiling density of DNA loops; Altered superhelicity might function as a sensor of UVB induced DNA lesions, and has special role in the regulation of DNA repair. We wanted to study the changes of supercoiling of DNA loops at different time-points after UVB exposure by a laser scanning cytometry (LSC) method and Halo assay. After design and perform some pilot experiments it turned out these experiments required to much mRNA quantity, which we was unable to managed (financed). Although the previous results were promising, we had to give up this project until the in vitro mRNA synthesis is getting more economic.

Purinergic receptors.

In the context of purinergic receptor signaling, our previous study has proved that $P2X_7$ receptor degradation by UVB irradiation can be associated with the lower induction of apoptosis in the UVB irradiated keratinocytes[21]. We wanted to determine the expression level of metabotropic and ionotropic **purinergic receptors and also intracellular calcium ion level** after photolyase mRNA transfection and UVB irradiation in human keratinocytes at mRNA and protein levels. After some very basic experiments we were not able to study this field due to a personal reason. Instead of concentrating of extracellular ATP we put more effort to investigate the intracellular energy balance as could be seen at point 2.2.

3. *In vivo* delivery of mRNA into the skin

The in vitro synthesized photolyase mRNA transfection theoretically would be an ideal method to protect the skin from the harmful effect of excessive UVB irradiation, but need to be delivered to the living skin cells.

3.1 Generating mRNA **nanoparticles** containing marker mRNA.
One of our OTKA research partner group have previously shown that chitosan and poly-gamma-glutamic acid in appropriate circumstances form nanoparticles, and when these particles bind folic acid folate receptor over-expressing cells are able to internalize them without decrease in cell viability. Therefore, this system is capable of targeted molecule delivery. As cancer cells frequently over-express the folate receptor, these nanoparticles may be used for early detection of melanoma metastases, by targeted delivery of contrast agents for more accurate MRI. Three melanoma cell lines were tested, WM35 (primary non-metastatic), WM983A (primary, metastatic to lymph nodes) and WM983B cells (from lymph node metastasis). Using confocal microscopy, we analyzed the folic acid containing nanoparticle uptake into cells. In the tested cell lines no nanoparticle internalization was observed. To see the potential viability of this approach in vivo, we also performed immunohistochemistry analysis of folate receptor (anti-FRalpha antibody) in primary melanoma, cutaneous metastatic melanoma and melanoma lymph node metastasis. Although folate receptor positivity was seen in macrophages surrounding the tumor cells, melanoma cells consistently failed to show staining.

3.2 Electroporation
Since our studies with nanoparticles were unsuccessful, additional experiments were carried out using ***in vivo* electroporation**. For optimization of the method, intradermal GFP plasmid transfection was carried out in the epidermis of adult mice. Immunohistochemistry was performed on the mouse skin samples, and show successful transfection of the marker plasmid, but sufficient expression in the epidermal basal cell layer was not observed. For optimization of the method, transfection of nucleoside-modified mRNA encoding GFP was carried out in the epidermis of adult mice avoiding invasive procedures including injections. Using non-invasive, in vivo electroporation with low (50V) –and high (200V) voltage pulses exposure as well as square wave pulses, we transfected microgram quantities (5 ug) of pseudouridine-modified mRNA encoding GFP into skin of adult mice. Immunohistochemistry was performed on the mouse skin samples but sufficient protein expression was observed neither in the epidermal basal cell layer nor in other layers of the skin.

3.3. Optimization of delivery of modified mRNAs into keratinocytes using **liposomal**

Currently, the ionizable lipid nanoparticles seem to be the best tool for delivering of in vitro synthesized mRNA, according to the study published by an American research team[11]. Nevertheless, there has not yet been suitable non-invasive delivery system to address in vitro synthesized mRNA into target cells or tissues. The ionizable lipid nanoparticles are very expensive and there are several types of nanoparticles. Our laboratory facilities do not allow us to optimize and try them to deliver in vitro synthesized mRNA into living skin.

EQUIPMENT

2 equipments were purchased:

one for the histopathology preparation of the samples a **cooling plate** (the old was out of order) and an **ice machine** for molecular biological studies.

PARTICIPATION IN SCIENTIFIC CONFERENCES

OTKA gave us the possibilities to participate in several scientific conferences, where we were able to present our work as posters or lectures and also be able to discuss them with other scientists. The personal meetings are very important for future possible international cooperation.

List of dates, attendees and conferences financed partly or totally by OTKA:

2016. April 06-09, Eva Remenyik prezented lecture Skin Care on Organtransplant Recipient in Europe (SCOPE) TelAviv, Israel

2016 June 20-25 Emese Gellen participated on the Photobiology school organized by the European Society of Photobiology in Bressanone, Italy

2016 Sept 6-10 Eszter Janka and Csaba Hegedűs presented posters on European Society for Dermatological Research

2016 September 27- October 02. Emese Gellén and Éva Remenyik participated on the 25th EADV Congress in Vienna with posters and lectures.

PhD degree

2 researchers have defended their PhD thesis from the research group

Eszter Emri 2016

Gabor Boros 2016

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