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The role of UVB-induced acute DNA damage in the regulation of mitochondrial function

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The Examination will be organized online at 11:00 on 2nd July, 2021.

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1. INTRODUCTION

Out of the UV spectrum that reaches the surface of the Earth UVB was demonstrated to have the greatest DNA damaging effect due to the formation of cyclobutane pyrimidine dimers (CPD). In mammalian cells, the repair of these photoproducts is accomplished exclusively by the complex yet slow nucleotide excision repair (NER). In lower taxonomic classes, a much more efficient repair mechanism also prevails, called photoreactivation. Photoreactivation uses the light-dependent enzyme, photolyase, to rapidly repair photolesions. However, this repair pathway disappeared from placental mammals during evolution. The UVB-induced DNA damage response involves the activation of signalling pathways that regulate DNA repair, redox homeostasis, degradation of damaged cellular components, metabolic changes, and mitochondrial function. In addition, UVB-induced reactive oxygen species (ROS) are regulators of several (patho)physiological processes. Although the role of UVB-induced apoptosis, inflammation, and carcinogenesis are well characterized, the effects of UVB on cellular metabolism and mitochondrial activity are currently unexplored. The earliest DNA damage responder protein, poly(ADP-ribose) polymerase 1 (PARP1), may link the UVB-induced acute DNA damage with functional changes in mitochondria. Therefore, beyond modulating DNA repair and maintaining genomic integrity, PARP1 plays a key role in regulating nucleus-mitochondria anterograde signalling.

1.1. UV radiation

UV rays can be subdivided into 3 different classes based on their wavelength properties: UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm). UVC radiation is completely filtered out by the ozone layer, so it does not reach our skin. UVB radiation affects the epidermis, the upper layer of our skin. The energy of UVA radiation is absorbed by the dermis as well. Each UV spectrum also differs in its mode of action. UVC radiation has the greatest genotoxic effect, involving the formation of cyclobutane pyrimidine dimers (CPD) and single- and double-strand DNA breaks. Similar to UVC, UVB directly damages the DNA; however, UVB mainly causes the formation of covalent crosslinks, including CPDs, (6-4) photoproducts (6-4)PPs, and Dewar isomers [photoisomerization products of (6-4)PPs]. UVA radiation causes oxidative damage mainly through the induction of reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation, the release of inflammatory cytokines, single-strand DNA breaks, and 7,8-dihydro-8-oxoguanine (8-OH-dG) lesions.

UV radiation causes a wide variety of biological effects. Acute effects include the synthesis of vitamin D, the appearance of apoptotic keratinocytes (sunburn cells), and metabolic changes. These acute effects are manifested clinically in the form of erythema, blister formation, hyperpigmentation, and hyperalgesia. Acute changes may develop into epidermal hyperplasia after days. Both acute and chronic UV-induced immunosuppression may develop. Skin aging and carcinogenesis are considered long-term effects. Since UVC and a large part of UVB are filtered out by the stratospheric ozone layer, 90-95% of the UV rays reaching the Earth's surface is UVA and the remaining 5-10% is UVB. Nevertheless, UVB is thought to be responsible for mediating most of the biological effects of the UV spectrum. UVB-induced photolesions lead to the appearance of UVB-specific signature mutations such as C> T and CC> TT transitions. These kinds of mutations have been observed in keratinocyte-derived skin tumours [basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)], as well as in pre-cancerous epithelial lesions (solar keratosis) and even in chronically photodamaged skin.

1.2. Nucleotide excision repair (NER)

NER is a complex repair mechanism, which is responsible for the removal of UVB-induced photoproducts in both prokaryotes and eukaryotes. NER consists of more than 30 proteins subdivided into global genome repair (GG-NER) and transcription-coupled (TC-NER) repair mechanisms based on differences in DNA damage recognition. The GG-NER sub-pathway is initiated by the DNA damage-binding 2 (DDB2) protein and repairs the heterochromatic regions. In contrast, the trigger signal for TC-NER is the stalled RNA polymerase II at the site of the lesion. Thus, TC-NER repairs actively transcribed DNA segments. The subsequent steps are similar for both sub-pathways. Once the DNA-binding proteins recognized the lesion-induced conformational change, helicases unwind the DNA strands around the lesion. This is followed by the excision of approximately 24-32 nucleotide segment containing the lesion. The resulting gap is filled by DNA polymerases and the ends are joined by DNA ligases. Genetic defects in NER are rare, but cause the development of serious diseases, such as Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS), and Trichothiodystrophy (TTD).

1.3. Photoreactivation

Throughout the world, from bacteria to placental mammals, another repair mechanism also prevails for the removal of UVB-induced photoproducts, called photoreactivation. Photoreactivation is catalysed by DNA photolyases, which are called CPD-specific or (6-4) photoproduct-specific photolyases based on their substrate specificity. Both photolyases are

approximately 55 kDa water-soluble monomeric flavoproteins, which contain reduced $FADH^-$ as a prosthetic group and methyl tetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyldeazariboflavin (8-HDF) and, on rare occurrences, pterin, as light-harvesting antenna. After 300–500 nm light exposure, the energy absorbed by the light-harvesting antennas is transferred to the reduced $FADH^-$ by a FRET mechanism. The excited $FADH^{-*}$ then transmits an electron to the CPD, which breaks the covalent bond connecting the two bases and the electron returns to the FADH° to regenerate the active $FADH^-$.

1.4. The role of reactive oxygen species (ROS)

Reactive oxygen species are the by-products of physiological processes inside cells. ROS can be formed in various subcellular compartments due to the activity of different enzymes and mitochondria, in particular, contribute to the formation of free radicals. Exogenous environmental effects, including UV radiation, are also significant ROS-producing factors. Since free radicals play a central role as secondary messengers in the regulation of various signalling pathways, cells maintain pro- and antioxidant balance. The epidermis has a number of chromophores that cause the formation of free radicals by transferring the energy absorbed by UV to biomolecules or directly to molecular oxygen. The skin contains several enzymes and antioxidants that play essential roles in preventing the harmful effects of ROS. If the redox balance is upset due to a lack of antioxidants and/or excessive ROS production, oxidative stress occurs. However, an increase in the level of reducing equivalents or excessive activation of antioxidant systems can deplete the cellular free radical pool and create anti-oxidative or reductive stress, which can paradoxically lead to phenotypic changes similar to oxidative stress by feedback regulation.

1.5. Mitochondrial dynamics and quality control

Mitochondria are highly dynamic organelles that undergo coordinated fission and fusion events to control their shape, size, and mass, collectively referred to as “mitochondrial dynamics”. Changes in mitochondrial dynamics serve to maintain optimal mitochondrial function, regulate nutrient supply, and fine-tune the metabolic needs of the cell. Mitochondrial fragmentation is often associated with deterioration of mitochondrial function, as this morphological condition is observed predominantly during severe stress and apoptosis. Mitochondrial fusion allows the distribution of mitochondrial matrix components within the network, promotes complementation and homogeneity between mitochondria, increases mitochondrial activity, provides protection against autophagy, and is generally associated with cell survival. The main

mediators of mitochondrial dynamics: mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optical atrophy 1 (OPA1) support mitochondrial fusion and dynamin-related protein 1 (Drp1) is responsible for fission. All four of these proteins are members of the guanosine triphosphatase (GTP) family. Mitochondrial biogenesis and mitochondrial selective autophagy (mitophagy) play key roles in the regulation of mitochondrial quality control. They regulate mitochondrial content in a coordinated manner, according to the cellular metabolic state and external and internal stress. Mutations in the genes encoding fission and fusion machinery components, imbalance between mitochondrial proliferation and degradation, deregulated mitochondrial quality control cause the development of serious diseases including tumours, neurodegenerative diseases, and metabolic syndromes.

1.6. Autophagy

Autophagy is a catabolic process that is essential for maintaining cellular homeostasis through lysosomal degradation of damaged cytoplasmic components, abnormal protein aggregates, and old or damaged organelles. Three main types of autophagy function in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy. Autophagy may be non-selective or selective. Non-selective macroautophagy randomly engulfs and ingests any damaged cytoplasmic components. Selective autophagy specifically degrades damaged organelles. By breaking down and recycling intracellular components, autophagy generates new building blocks for anabolic processes on one hand and on the other hand provides energy for catabolic pathways. Thus, autophagy plays a central role in the regulation of carbon and nitrogen metabolism and provides metabolic plasticity to cells.

1.7. The regulation of mitochondrial metabolism

The best-characterized role of the mitochondria is the production of adenosine triphosphate (ATP) via the oxidation of various substrates (sugars, amino acids, and fatty acids). The respiratory chain consists of five complexes: complex I (NADH ubiquinone oxidoreductase), complex II (succinate ubiquinone oxidoreductase), complex III (ubiquinol cytochrome C reductase), complex IV (cytochrome C oxidase), and complex V (F_0F_1 ATP synthase). Electrons flow through the respiratory chain from Complex I to IV, while protons are being pumped out from the mitochondrial matrix into the intermembrane space. Due to the proton gradient formed between the two sides of the inner membrane, the protons flow back through complex V, and energy in the form of ATP is generated from ADP and inorganic phosphate (Pi). Mitochondrial metabolism is regulated both directly and indirectly by a number of

proteins, including Sirtuin 1 (SIRT1), Ataxia telangiectasia mutated (ATM), AMP-activated protein kinase (AMPK), and p53, which support mitochondrial biogenesis and oxidative metabolism and inhibit anabolic processes. These proteins associate with the mitochondria and induce mitochondrial translocation of their substrates. Among the cellular nutrient-sensing systems, protein kinase B (AKT) and the mTOR pathways regulate anabolic processes required for cell growth and proliferation. These pathways also positively regulate mitochondrial biogenesis and oxidative metabolism. PARP1, which uses NAD^+ for its enzymatic activity, plays a key role in the regulation of the cellular NAD^+ pool, and persistent activation of PARP1 is commonly associated with functional deterioration of mitochondria in Cockayne syndrome, Xeroderma Pigmentosum, and Ataxia Telangiectasia. However, reduced NAD^+ levels may quickly recover after transient PARP1 activation and mitochondrial dysfunction does not occur. Furthermore, PARP1-induced NAD^+ depletion may shift the metabolic balance toward oxidative phosphorylation, which is essential for cell survival. Each metabolic protein can inhibit or promote the activity of the others, influencing the net mitochondrial energy production, exemplifying the versatility and complexity of these metabolic sensors.

2. OBJECTIVES

UVB radiation-induced DNA damage has emerged as an important factor in the development of acute inflammation (dermatitis solaris), keratinocyte skin cancers, and skin aging. A growing number of scientific reports describe signalling between the nucleus and mitochondria; thus UVB-induced DNA damage may also be involved in fine-tuning mitochondrial function. PARP1, the most characterized member of the PARP superfamily, may play a role in the regulation of signalling pathways between the nucleus and mitochondria induced by UVB. In the first half of our work, we investigated the role of PARP1 protein in the regulation of UVB-induced DNA repair, mitochondrial biogenesis, mitochondrial morphology and metabolism, and autophagy following physiologically relevant doses of UVB irradiation in human HaCaT keratinocytes in particular. In addition, we aimed to identify the pathways involved in the metabolic changes induced by UVB irradiation and PARP1 inhibition.

In mammals, the repair of UVB-induced cyclobutane pyrimidine dimers (CPDs) is accomplished by the nucleotide excision system. In lower taxonomic classes, a much more efficient repair mechanism, called photoreactivation, is employed to rapidly repair the covalent bond between two pyrimidine bases using a photolyase enzyme. However, this repair mechanism has disappeared from placental mammals during evolution. Although CPDs are the most important lesions mediating UVB-induced DNA damage, experimental verification of CPD-dependent effects is cumbersome and there is no scientific data concerning the role of CPDs in the regulation of mitochondrial function. We were able to map the UVB-induced CPD-dependent processes by transfecting keratinocytes with an in vitro synthesized N1-methyl pseudouridine-modified CPD-specific photolyase encoding mRNA. In the second half of our work, we aimed to investigate the role of CPDs and reactive oxygen species (ROS) in the regulation of DNA damage response, mitochondrial morphology and function, lipid droplet biogenesis, autophagy, keratinocyte differentiation, and mitochondrial substrate utilization.

3. MATERIALS AND METHODS

3.1. Cell culture

The human immortalized HaCaT keratinocyte and CHO-K1 cell lines were cultured in 4500 mg/L high glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and sodium pyruvate containing 10% heat-inactivated fetal bovine serum (FBS) and 0.5% antibiotic/antimycotic solution. Neonatal human epidermal keratinocytes (NHEK) were cultured in Epilife Medium with 60 µM calcium supplemented with Human Keratinocyte Growth Supplement and 0.5% antibiotic/antimycotic solution. Cells were maintained in a T75 flask at 37 °C with 5% CO_2 tension.

3.2. UVB irradiation and cell treatments

Cells were harvested with 1x trypsin-EDTA and seeded into 12, 24, or 96-well cell culture plates. For UVB irradiation, the medium was removed, cells were washed twice with prewarmed Dulbecco's phosphate-buffered saline (DPBS), and then irradiated depending on the size of the cell culture plate in 400, 300, or 30 µL DPBS with a total dose of 20 or 40 mJ/cm^2 UVB. UVB irradiation was performed with two broad-band UVB tubes (TL-20W/12 RS; Philips, Eindhoven, The Netherlands). Control cells were covered with tin foil. Following UVB irradiation, DPBS was replaced with complete medium and cells remained in the incubator for an additional 24 hours. The proper dose of UVB was checked in each case with a UVX digital radiometer.

3.3. CPD-specific mRNA-based photolyase transfection and photoreactivation

The mRNA encoding the *in vitro* synthesized CPD-specific photolyase enzyme from Potorous tridactylus was prepared with a linearized plasmid (pTEV-CPD-PL-A101). The mRNA was generously gifted by our collaboration partners Prof. Katalin Karikó and Dr. Gábor Boros (BioNTech RNA pharmaceuticals GmbH, BioNTech AG). The purity of the mRNA was analysed by agarose gel electrophoresis. Photolyase mRNA transfection was performed in a 24-well plate. Serum- and antibiotic-free Epilife medium (400 µL) was mixed with 500 ng of mRNA, 1 µL of Lipofectamine Plus, and 2 µL of Lipofectamine LTX. The complex was incubated with the cells for 2 hours. UVB irradiation was performed in 300 µL DPBS. Photoreactivation was performed in the same solution with two fluorescent tubes (Sylvania Standard F18W/54-765 Daylight 6500K) for 30 min. Control cells were covered with tin foil. Cells were incubated in complete DMEM or Epilife for an additional 24 hours in the incubator.

3.4. Gene silencing

On-target plus SMARTpool PARP1 siRNA specific for PARP1 protein was purchased from Dharmacon Research, Inc. Non-specific siRNA was used as a control for the experiment. Cells were cultured in antibiotic-free DMEM containing 10% FBS. The DharmaFECT transfection reagent and siRNA mix were incubated together for 20 min and then added to the cells at a final siRNA concentration of 50 nM. After 48 hours, the cells were irradiated with UVB and incubated in complete DMEM for an additional 24 hours.

3.5. Cell viability

Cell viability was determined using a Dead Cell Apoptosis Kit according to the manufacturer's instruction. Stained cells were analysed by flow cytometry on a FACSCalibur instrument. CellQuest Pro software 5.2 and Flowjo Single Cell Analysis v10.0.7 were used for data collection and evaluation.

3.6. Cell proliferation

Cell proliferation was determined using a clonogenic assay. Cells were harvested after UVB irradiation with 1x trypsin-EDTA solution and plated in 100 mm Petri dishes in equal numbers of cells ($5x10^4$ cells/well). Ten days later, keratinocytes were washed with DPBS, fixed with 100% methanol, and stained with May-Grünwald-Giemsa solution.

3.7. Cell cycle analysis

To determine cell cycle progression, cells were trypsinised, fixed with 96% ice-cold ethanol for 10 min, and then permeabilized with 0.1% Triton X-100 for 10 min. After washing twice with 1x DPBS, cells were incubated in DPBS containing 0.5 mg/mL RNase for 1 hour at 37 °C. Propidium iodide at a final concentration of 20 µg/mL was used to label the DNA content of the cells. The DNA content of the samples was determined by flow cytometry.

3.8. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutation assay

CHO cells were maintained in hypoxanthine-aminopterin-thymidine (HAT) medium for one week. After UVB irradiation, cells were grown in complete DMEM for one week, and then $5x10^4$ cells in 100 mm Petri dishes were further cultivated for an additional 10 days in selection medium containing 5 µg/mL 6-thioguanine (6-TG). Cell fixation, permeabilisation, and staining were performed similarly to the clonogenic (cell proliferation) assay described earlier.

3.9. CPD-specific enzyme-linked immunosorbent assay (ELISA)

DNA was isolated with Purelink Genomic DNA Mini Kit according to the manufacturer's instructions. DNA was denatured for 10 minutes at 100 °C and then allowed to stand on ice for 15 minutes. DNA (15 ng) was added to each well of a 96-well plate pre-coated with 0.003% protamine sulphate and DNA was left to dry overnight at 37 °C. The plate was washed three times with PBS containing 0.05% Tween-20 (PBST) and incubated in 2% FBS blocking buffer for 30 minutes at 37 °C. Samples were incubated at a 1:1000 dilution with mouse anti-CPD (clone: TDM2) antibody at 37 °C for an additional 60 minutes. After washing with PBST, horseradish peroxidase-conjugated anti-mouse IgG secondary antibody was added to the wells at 1:3000 dilution and the mixture was incubated at 37 °C for 30 minutes. After incubation, the plate was washed with PBST and the solution was changed to citrate phosphate-containing equilibration buffer (51.4 mM Na_2HPO_4, 24.3 mM citric acid monohydrate, pH 5.0). The buffer was removed and a citrate phosphate solution containing 0.0063% H_2O_2 and 0.04% o-phenylenediamine substrate was added to the samples. After 30 min, the reaction was stopped with 2 M H_2SO_4 solution and the absorbance was determined at 492 nm with an ELISA microplate reader.

3.10. Real-time PCR

Total RNA was isolated by guanidine isothiocyanate-phenol-chloroform extraction. The concentration and purity of the RNA samples were analysed with a NanoDrop spectrophotometer. Genomic DNA contamination was removed from the samples by DNase I treatment. Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit as described by the manufacturer. The PCR product was monitored by the increasing fluorescence intensity of the SYBR green dye on a 384 well optical plate using a Lightcyler 480 II instrument. Gene expression was determined by the $2^{-\Delta CT}$ method. Expression values were normalised to the housekeeping genes, succinate dehydrogenase (SDHA) and phosphoglycerate kinase (PGK1).

3.11. Western blot

Cells were harvested with 1x trypsin-EDTA solution and lysed on ice in RIPA buffer containing a 1:1000 protease inhibitor cocktail. After centrifugation at 1500 rpm for 5 minutes at 4 °C, the protein content of the supernatant was determined using a BCA Assay Kit. The lysate was boiled in 5x loading buffer at 100 °C for 10 minutes. Samples were separated on a SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane and then blocked

in TBST containing 5% BSA. Membranes were incubated with primary antibody at 4 °C overnight. Following the washing steps, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody for one hour at room temperature. Secondary antibody binding was visualized with Pierce ECL Western Blotting Substrate or SuperSignal West Femto Maximum Sensitivity Substrate. Bands were quantified with the open-source ImageJ v1.52a software. Proteins were normalised to the loading control, β-actin.

3.12. Electron microscopy

Keratinocytes were harvested by trypsinisation, washed with DPBS, and centrifuged at 1500 rpm. The pellet was fixed for 2 hours in 0.1 M cacodylate buffer containing 3% glutaraldehyde and 5% sucrose. Osmification occurred in 1% osmium tetroxide (OsO_4) and samples were dehydrated using an increasing alcohol series. Samples were embedded by Durcupan araldite treatment, and, after encapsulation, ultrathin sections were made with a Leica EM UC7 ultramicrotome. Standard contrasting was performed with uranyl acetate and Reynolds lead citrate solution. High-resolution transmission electron microscopy (TEM) images were captured with Jeol JEM 1010 electron microscope and software.

3.13. Confocal microscopy

Cells were grown on a glass plate and stained with the following reagents depending on the cellular compartment to be detected at 37 °C for 30 minutes: for mitochondrial network visualization, 100 nM Mitotracker Red CMXRos; for lipid droplets detection, 1 µg/mL Bodipy 493/503 or 30 µL Adipored in 1 mL DPBS was added to the medium. Samples were washed twice with DPBS, fixed with 3.7% paraformaldehyde at room temperature for 20 min, permeabilized with 0.2% Triton X-100, blocked with 1% BSA for 1 h, and then incubated with the primary antibody in a humid chamber at 4 °C overnight. Autophagosomes were visualized by Alexa Fluor 488-conjugated LC3A/B antibody at 1:50 dilution and Keratin 1 (K1) antibody was used at a 1:750 dilution for K1 detection. On the next day, samples were washed with DPBS and incubated with Alexa Fluor 488-conjugated anti-rabbit secondary IgG antibody for one hour at room temperature for K1 expression. Samples were stained with mounting medium containing DAPI and analysed using an Olympus FV3000 microscope with 60x oil immersion objective.

3.14. Mitochondrial mass

Samples were incubated with 100 nM Mitotracker Green at 37 °C for 30 minutes. Cells were washed with DPBS twice, harvested by trypsinization, and immediately placed on ice. The intensity of Mitotracker Green was analysed by flow cytometry.

3.15. Mitochondrial membrane potential

Mitochondrial membrane potential was determined using 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] or 100 nM Mitotracker Red CMXRos. Samples were incubated with the dyes at 37 °C for 30 minutes. Cells were washed with DPBS twice, harvested with 1x trypsin-EDTA solution, and immediately placed on ice. The intensity of Mitotracker Red CMXRos was determined by flow cytometry.

3.16. Reactive oxygen species (ROS)

The determination of ROS production was performed similarly to the previous two methods. The ROS dye used here, dihydroethidium, was applied at a final concentration of 10 µM and fluorescence was measured by flow cytometry.

3.17. Mitochondrial reactive oxygen species (mtROS)

Samples were incubated with 1 µM MitoSOX Red at 37 °C for 10 minutes. Cells were washed with DPBS twice, harvested by trypsinization, and immediately placed on ice. The fluorescence intensity of MitoSOX Red was measured by flow cytometry.

3.18. Mitochondrial DNA isolation

Cells were harvested by trypsinization, washed with DPBS, and pelleted at 1500 rpm for 10 minutes. Mitochondrial DNA was isolated from the pellet using the Mitochondrial DNA Isolation Kit as described by the manufacturer.

3.19. Citrate synthase (CS) activity

The CS activity of the keratinocytes was determined using a Citrate Synthase Assay Kit according to the manufacturer's protocol. Changes in optical density (OD) at 412 nm were determined using the kinetic program of the ELISA microplate reader. OD values were normalized to total protein content.

3.20. Analysis of mitochondrial substrate preference with Seahorse XF Mito Fuel Flex Test Kit

One hour before the assay, the complete DMEM on the cells was replaced with unbuffered DMEM containing 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 mM D-glucose. Cells were equilibrated for one hour in a non-CO_2 incubator. Mitochondrial oxidation of glutamine, fatty acids, and pyruvate was measured with the injection of BPTES, Etomoxir, and UK-5099, respectively, according to oxygen consumption values measured with an XF96 Extracellular Flux Analyzer.

3.21. Measurement of glycolysis and oxidative phosphorylation

One hour prior to the assay, the complete DMEM on the cells was replaced with unbuffered DMEM containing 10 mM D-glucose. Cells were equilibrated for one hour in a non-CO_2 incubator. After four baseline measurements, oligomycin, FCCP, and antimycin A were injected. Oxygen consumption (OCR) reflects oxidative phosphorylation and extracellular acidification rate (ECAR) represents glycolysis. All OCR and ECAR values were normalized to total protein content.

3.22. Endogenous and exogenous fatty acid oxidation

Immediately after UVB irradiation, cells were maintained in substrate-limited medium for 23 hours. The substrate-limited medium was replaced with fatty acid oxidation (FAO) medium 45 minutes before the assay. FAO assay medium (135 µL) was added to each well and the plate was kept in a non-CO_2 incubator for an additional hour. To discriminate endogenous and exogenous fatty acid oxidation, 30 µL of XF Palmitate-BSA FAO Substrate or BSA and Vehicle or 40 µM Etomoxir were added to the respective samples. After the four baseline measurements, oligomycin, FCCP, and antimycin A were injected. All OCR values were normalized to total protein content.

3.23. ATP content

Cells were washed with PBS and spun at 1500 rpm. After aspirating the supernatant, the total ATP content of the pellet was determined using an ATP Colorimetric/Fluorometric Assay Kit according to the manufacturer's instruction. OD values were normalized to total protein content.

3.24. NAD^+ content

Cells were washed with PBS and spun at 1500 rpm. The NAD^+ content of keratinocytes was determined with a NAD/NADH Quantitation Colorimetric Kit according to the manufacturer's description. OD values were normalized to the total protein content.

3.25. Statistical analysis

The normality of the population was determined by the Kolmogorov–Smirnov test. If the population did not show normal distribution, values were transformed logarithmically or by the Box-Cox method. The frequency of each mitochondrial morphological subtype was determined by Chi^2 or Fisher's exact test. Significant differences between the multiple groups were determined by ANOVA followed by Sidak's or Dunnett's post-hoc tests. All data are presented as mean ± standard error of the mean (SEM). The results were considered significant at $p < 0.05$.

4. RESULTS AND DISCUSSION

We aimed to reveal the relationship between UVB-induced DNA damage and altered cellular metabolism. Persistent DNA damage and defects in the DNA repair machinery result in mitochondrial dysfunction. This bioenergetic deterioration is observed in genetic diseases (Ataxia telangiectasia, Xeroderma Pigmentosum, Cockayne syndrome) where genes essential for DNA repair are damaged. Although the roles of UVB-induced DNA damage response, apoptosis, photoaging, and carcinogenesis are well characterized, convincing data on how UVB affects mitochondrial function are still missing. Investigating the morphological and functional alterations of mitochondria by modifying the efficiency of DNA repair has been the central element of my research. A descriptive study is not always suitable for exploring a physiological process, like the UVB-induced stress response and mitochondrial signalling. In contrast, if we study cellular processes by modelling biologically relevant impacts, we can gain valuable information on cellular function. For this, we used a drug (veliparib) that inhibits PARP1, a protein playing a prominent role in DNA repair. Veliparib is approved by the FDA (Food and Drug Administration) for the treatment of various solid tumours. An increase in the UVB-induced DNA damage was expected after PARP1 inhibition. Reducing DNA damage was another central element of our research. To this end, keratinocytes were transfected with *in vitro* synthesized CPD-photolyase-encoding mRNA from Pototrous tridactylus. Photolyase mRNA was translated into protein that rapidly repairs UVB-induced CPDs. Overexpression of photolyase was necessary because the enzyme has disappeared during evolution from placental mammals, including humans. Although the two experimental setups are not simply opposites, we were able to investigate the role of UVB-induced DNA damage in the regulation of mitochondrial function in a physiologically relevant cell type and UVB dose.

4.1. DNA damage

Rapid accumulation of poly(ADP-ribose) polymer was observed after UVB irradiation in a dose-dependent manner. Among the members of the PARP superfamily, PARP1 has predominant poly(ADP-ribose) polymerase activity. PARP1 contributes about 85-90% of the total PARylation signal in cells. ABT-888 (veliparib) significantly reduced PARylation even after UVB irradiation. Thus, PARP1 is considered to be the main mediator of UVB-induced PAR formation. Lack of PARylation significantly reduced the removal of CPDs, indicating impaired NER activity. Defective NER after PARPi enhanced UVB-induced G_2/M cell cycle block, which is a typical example of acute DNA damage. In light of these data, it is not surprising that inhibition of PARP1 after UVB irradiation significantly reduced cell

proliferation and viability. Our results are consistent with experimental data and clinical experience with PARP inhibitor (rucaparib, olaparib, stenoparib, niraparib) induced photosensitive reactions. Besides CPDs, the accumulation of (6-4)photoproducts can be detected after UVB irradiation. In contrast to CPDs, these photolesions are rapidly repaired by the nucleotide and base excision repair. For this reason, CPDs are considered to be the major lesions mediating UVB-induced DNA damage. However, experimental verification of CPD-dependent effects is still burdensome and this area in photobiology is almost completely unexplored. In our unique experimental model system, we were able to investigate these CPD-dependent effects by overexpressing the CPD-specific photolyase enzyme. Photolyase, activated by visible light, rapidly removed CPDs from the nuclear and the mitochondrial genome. The decreased UVB-induced cell cycle block, keratinocyte proliferation, and viability were completely restored by photolyase activation, emphasizing the key role of CPDs in mediating UVB-induced DNA damage. Thus, contrary to PARP1 inhibition, we observed significantly different effects on UVB-induced DNA damage in this experimental setup.

4.2. NAD^+ depletion and the activation of NAD^+ consuming enzymes

PARP1 is one of the earliest responders to DNA damage, playing a central role in the regulation of cellular NAD^+ stores. PARP1 is a very aggressive NAD^+ consumer. Some reports estimate that the total NAD^+ depletion by PARP1 is 50-80%. Therefore, it is not surprising that a significant UVB-induced decrease in total NAD^+ level was observed, even hours after PARP activation. The administration of the PARP inhibitor robustly increased cellular NAD^+ content. A similar change was detected after photolyase activation, where the NAD^+-depleting effect of UVB was completely absent. Surprisingly, removal of CPDs could only partially block PARP activation, raising the possibility that (6-4)photoproducts may regulate PARylation. The activation of another enzyme family, the sirtuins, may also account for UVB-induced NAD^+ consumption. Indeed, CPDs upregulated all members of the sirtuin family (SIRT1-7) after UVB, which may explain why CPD-dependent NAD^+ depletion of keratinocytes could be avoided by partially decreased PARP1 activity. Sirtuins with different subcellular localizations are involved in the regulation of various cellular processes, including metabolism and DNA repair. Our results show that, in addition to PARP1, SIRTs may also be involved in the regulation of NAD^+ levels post-UVB irradiation and may actively assist in the recognition of DNA damage and the coordination of DNA repair.

4.3. Mitochondrial fusion

Mitochondrial morphology changes dynamically according to environmental cues and metabolic needs of cells. The shape of mitochondria depends on the balance of fusion and fission and can range from rounded, well-separated forms to completely elongated, spaghetti-like, networked structures. In addition, mitochondrial quality control is coordinately regulated by the rate of mitochondrial synthesis (mitochondrial biogenesis) and degradation (mitophagy) l. Our results show that UVB irradiation and PARP inhibition increased the number of mitochondria, total mitochondrial area and mass, and MTCO1 protein expression. We also detected gene expression upregulation in transcription factors that control mitochondrial biogenesis. In the absence of DNA damage (photolyase activation), no changes indicative of UVB-induced mitochondrial biogenesis were observed. Similarly, elevated mitochondrial biogenesis and biogenesis-related functional changes were observed after hydrogen peroxide and etoposide treatment and ionizing and UVC radiation, supporting the relationship between acute DNA damage and mitochondrial biogenesis. We observed fusion of the mitochondrial network post-UVB irradiation accompanying mitochondrial biogenesis and this mitochondrial fusion was even more prominent after PARP1 inhibition. If CPDs were removed by the photolyase enzyme, mitochondrial morphological changes were absent. Changes in mitochondrial dynamics accompany DNA damage and are cell type-specific. Similar mitochondrial fusion has been observed following cycloheximide, actinomycin D, and UVC irradiation. This mitochondrial fusion is referred to as stress-induced mitochondrial hyperfusion (SIMH) and is part of the protective response. We also interpret the UVB-induced mitochondrial fusion and biogenesis as a protective mechanism. Fusion promotes the exchange of DNA and metabolites (ADP, NADH, $FADH_2$) between two or more partially damaged mitochondria, whereby keratinocytes try to maintain oxidative phosphorylation and alleviate DNA damage. This is supported by the fact that these changes were detected in UVB-irradiated keratinocytes in a dose-dependent fashion. Inhibition of PARP1 increased CPDs and photolyase activation reduced the level of CPDs, confirming the dependence of mitochondrial fusion and biogenesis on UVB-induced acute DNA damage.

4.4. Cellular and mitochondrial metabolism

Several aspects of DNA damage are strongly linked to mitochondrial and metabolic activities of cells. A number of proteins are involved in the recognition of DNA damage and the regulation of DNA repair. These proteins can directly or indirectly regulate mitochondrial energy production at the cellular and whole-organism levels. Mutations in these proteins occur

in Werner syndrome (WS), Ataxia Telangiectasia (AT), Hutchinson–Gilford progeria (HGPS), and Cockayne syndrome (CS). A common feature of these diseases is that they are caused by the functional decline of a protein involved in the DNA-damage response. PARP1, AMPK, and NAD^+ play a central role in mediating UVC-induced mitochondrial changes, as observed in NER deficient $CSA^{-/-}$, $XPA^{-/-}$ mutant mice. Similar to UVC-induced acute DNA damage, we detected mitochondrial membrane hyperpolarization and increased glycolysis, TCA cycle, oxidative phosphorylation, and fatty acid oxidation after UVB irradiation. After chemical inhibition of the protein of interest, we confirmed the role of ATM, AMPK, p53, AKT, and mTOR pathways (by western blot) in the regulation of OXPHOS and CPT1A, HADHA, ACADM proteins in modulating fatty acid oxidation. Several mitochondrial changes were enhanced by PARP1 inhibition, while CPD removal significantly reduced all mitochondrial phenotypic alterations, demonstrating the role of UVB-induced DNA damage in regulating mitochondrial metabolism. Although chronic DNA damage or a defect in DNA repair often leads to a decrease in energy homeostasis and permanent mitochondrial dysfunction, an acute, tolerable level of DNA damage results in increased mitochondrial metabolism that provides an adequate supply of energy for nucleotide excision repair. Several proteins that play key roles in NER require ATP for the recognition of DNA damage and binding to the DNA lesions. Thus, UVB-induced increases in mitochondrial function are part of an adaptive and anti-apoptotic response of cells. This is evidenced by the observation that inhibition of mitochondrial biogenesis or proteins involved in oxidative phosphorylation resulted in decreased cell viability after UVB irradiation. These data suggest that CPD-dependent activation of the ATM/ATR, AMPK, p53, AKT, and mTOR pathways may enhance the efficiency of nucleotide excision repair. In line with enhanced mitochondrial metabolism, the dependence of cells on pyruvate, fatty acids, and glutamine oxidation was increased in a CPD-dependent manner following UVB irradiation, but this increased substrate preference was not accompanied by an increase in total available supply, indicating that keratinocytes cannot switch freely and effectively between the three main mitochondrial energy sources after UVB irradiation. These changes were completely absent when CPDs were removed, suggesting that all three substrates are required to repair UVB-induced DNA damage. This is supported by the fact that glutamate, as an essential amino acid, is required for nucleotide biosynthesis and, thus, for DNA repair. Studies also report that fatty acid synthesis by PARP1 protein, beta oxidation, and oxidative phosphorylation provide reducing equivalents to aid DNA repair. Finally, pyruvate supplementation reduces DNA damage and induces the nuclear translocation of pyruvate kinase 2 (PKM2), which is involved in pyruvate metabolism, to promote repair of DNA lesions by stimulating homologous

recombination after H_2O_2 treatment and UVB irradiation. Overall, we demonstrated the substrate preference modulating effect of acute DNA damage and its role in enhancing mitochondrial activity, which can reciprocally react to promote DNA repair.

4.5. Autophagy and lipophagy

DNA damage-induced autophagy is well-known, whereby cells meet their bioenergetic demands via breaking down and recycling damaged cellular components and macromolecules in the lysosomes. Along with UVB-induced CPD accumulation, an increase in the number of $LC3^+$ vesicles (autophagosomes) was observed. Inhibition of PARP1 enhanced UVB-induced autophagy, while photoreactivation of photolyase abolished it, suggesting a role for CPDs in the regulation of autophagy. Mounting evidence also suggests that autophagy triggers a positive signal in the initiation of the GG-NER sub-pathway, that is, CPDs may promote their repair through the induction of autophagy. Selective degradation of mitochondria, termed mitophagy, was not observed after UVB irradiation. This can be explained by the UVB-induced mitochondrial fusion and mitochondrial membrane hyperpolarization to avoid mitochondrial dysfunction that would signal for the initiation of mitophagy. Surprisingly, in parallel with the induction of autophagy, an increase in the number of lipid droplets was detected in a CPD-dependent manner. Although the role of the DNA damage-induced lipid droplet biogenesis is hardly known, based on the autophagosome-lipid droplet colocalization, it is believed that the degradation of lipid droplets by autophagy (lipophagy) may provide free fatty acids for mitochondrial beta oxidation. In addition, free fatty acids released during the recycling of organelles surrounded by the intracellular membranes may be packed into lipid vesicles if not used in mitochondrial beta oxidation. Cells can also meet their demand for increased substrate utilization and mitochondrial activity by recycling damaged subcellular elements via autophagy. This is supported by the fact that inhibition of the ATM, AMPK, p53, PI3K1, and mTOR pathways reduced the expression of the general autophagy marker, LC3A/B, and the mitophagy-specific PARKIN protein. Thus, lack of autophagy/mitophagy and substrate availability results in the deterioration of mitochondrial quality control and the consequent mitochondrial activity. In addition, the role of autophagy in boosting mitochondrial metabolism is supported by the observation that when the oxidative metabolism-reducing chloroquine was used, it inhibited the autophagic flux. Consequently, the number of lipid droplets and autophagosomes were significantly increased. However, we must take into account that autophagy is also an energy-consuming process that requires an adequate supply of ATP. Therefore, the reciprocal regulation of autophagy-OXPHOS cannot be completely ruled out.

Surprisingly, no colocalization was observed between mitochondria and lipid droplets, suggesting that free fatty acids released from lipid droplets by cytoplasmic lipases are transported directly to mitochondria, which is much more efficient compared to direct lipid droplet-mitochondrial contact. Based on these results, we can conclude that UVB-induced autophagy is a key mechanism in regulating mitochondrial activity. In addition, lipid droplets, acting as a buffering system, play a central role in the regulation of mitochondrial metabolism, from which fatty acids are translocated into mitochondria to provide substrates for beta oxidation upon various stress conditions including UVB.

4.6. Keratinocyte differentiation

Keratinocyte differentiation is a special form of programmed cell death in which an extracellular high Ca^{2+}-gradient promotes the upregulation of the differentiation-specific keratins (KRT), filaggrin (FLG), loricrine (LOR), and involucrin (INV). Lipid droplets accumulate during the differentiation of keratinocytes. This lipid droplet accumulation contributes to the homeostasis and barrier function of the skin. Dysregulation of the differentiation process may initiate, among other things, the development of atopic dermatitis and play a role in the pathomechanism of psoriasis. Following UVB irradiation, an increase in the early differentiation marker, K1, was detected in a CPD-dependent fashion. We demonstrated the parallel and mitochondrial activity-dependent regulation of K1 and lipid droplets. Any inhibitor that counteracted the increased oxidative phosphorylation and substrate utilization of mitochondria simultaneously reduced lipid droplet accumulation and K1 protein expression. Our results are consistent with the literature demonstrating that high Ca^{2+} concentrations promote keratinocyte differentiation via the PI3K1/AKT and mTOR pathways. In addition, mitochondrial Ca^{2+} uptake enhances the activity of TCA cycle enzymes (pyruvate, isocitrate, and oxoglutarate dehydrogenase). Thus, increasing the oxidative metabolism of mitochondria promotes keratinocyte differentiation. Changes in lipid metabolism due to UVB irradiation also affect differentiation. The role of phosphatidylglycerol (PG) and diacylglycerol (DAG) in inducing differentiation has previously been demonstrated. Although the role of UVB-induced keratinocyte differentiation is still controversial, in line with the previously published data, we believe that differentiation is part of an adaptive response elicited by UVB to support cell survival and maintain tissue homeostasis and integrity.

4.7. Reactive oxygen species

Reactive oxygen species play a key role in the regulation of various physiological processes. Increased activity of peroxisomes, various oxidases, transient metal ions, protein folding in the endoplasmic reticulum, and thymidine and polyamine metabolism all contribute to the formation of free radicals. Nevertheless, mitochondria are considered to be the major source of ROS due to their oxidative phosphorylation. Twenty-four hours post-UVB irradiation, an increase in total and mitochondrial ROS production was detected, which was surprisingly abolished by CPD removal akin to ROS scavengers. The specific mitochondrial ROS scavenger, MitoTEMPO, was able to reduce the activation of several CPD-dependent pathways similarly to the general ROS scavengers, including GSH and NAC. Thus, glycolysis, oxidative phosphorylation, mitochondrial biogenesis, lipid droplet accumulation, autophagy induction, and keratinocyte differentiation were significantly decreased, suggesting that mitochondrial ROS production plays a prominent role in mediating UVB-elicited effects. Although some data report that antioxidants may reduce CPD formation, we have not been able to detect such a reduction in the case of any ROS scavengers. Considering these results, we believe that CPDs, by increasing mitochondrial activity, lead to secondary mitochondrial ROS production, which regulates multiple mitochondrial and extramitochondrial pathways in a ROS-dependent fashion.

4.8. Mutagenesis

Although the inhibition of PARP1 by veliparib treatment increased the UVB-induced photosensitive response, surprisingly, the lack of PARylation caused a significant reduction in the UVB-induced mutations. This phenomenon cannot be easily explained due to the regulatory mechanism of PARP1 on diverse DNA repair pathways (NER, BER, HR, etc.). However, this result is consistent with clinical experience. An increased risk of carcinogenesis was not observed in patients even after long-term use of veliparib. The role of mitochondria in initiating and promoting carcinogenesis is contradictory. The conventional view was that a decrease in mtDNA copy number, its altered sequence, and a deterioration in mitochondrial function were observed in cancer cells, which shifts the energy production of the cells towards anaerobic glycolysis. However, an increasing number of reports suggest that increased mitochondrial biogenesis and activity is a potential signal in mediating mutagenesis, whereby cancer cells with elevated mitochondrial activity are more resistant to pro-apoptotic signals like chemotherapeutic agents. The increased UVB-induced mutation rate was inhibited by photoreactivation of the photolyase, suggesting a key role for CPDs in initiating carcinogenesis.

Similar to CPD removal, inhibition of autophagy and the transport of mitochondrial substrates significantly reduced UVB-induced mutagenesis. Induction of autophagy and mitochondrial oxidation of pyruvate, fatty acids, and glutamine were CPD-dependent in our previous experiments. Therefore, increased mitochondrial activity and recycling of damaged cellular components may favour cell survival. These data suggest that, although increased mitochondrial activity supports DNA repair through the induction of oxidative phosphorylation, fatty acid oxidation, and glutaminolysis, the survival of potentially mutation-bearing cells may also be promoted in coordination with autophagy. Therefore, these pathways may also be relevant as therapeutic targets in UVB-induced mutagenesis.

5. SUMMARY

In our work, we demonstrate a novel link between UVB-induced acute DNA damage and altered mitochondrial morphology and metabolism. Inhibition of PARP1 increased UVB-induced mitochondrial biogenesis, fusion, and metabolism, while removal of CPD lesions via photoreactivation of the photolyase enzyme inhibited these mitochondrial phenotypes. In parallel with UVB-induced oxidative phosphorylation and fatty acid oxidation, we demonstrated the CPD-dependent utilization of the three major mitochondrial substrates, including pyruvate, fatty acids, and glutamine. We identified the role of the CPD-dependent activation of ATM, AMPK, p53, PI3K1/AKT, and mTOR pathways in the regulation of autophagy, lipid droplet biogenesis, keratinocyte differentiation, and mitochondrial activity. We have demonstrated that increased mitochondrial activity of cells promotes survival of UVB-irradiated keratinocytes. We have also shown the activation of a special form of autophagy, called lipophagy, and its role in regulating mitochondrial metabolism after UVB irradiation. In parallel with UVB-induced lipid droplet biogenesis, increased keratinocyte differentiation was observed, with a strong dependence on the mitochondrial activity of the cells. We have demonstrated the cellular NAD^+-depleting effect of UVB, which is due to the activation of PARP1 and the increased expression of Sirtuins. The activation of several mitochondrial pathways was observed that support the repair of DNA damage but may also promote the survival of mutation-bearing cells. CPDs as well as CPD-induced secondary mitochondrial ROS production play a prominent role in mediating several metabolic and extramitochondrial changes. Finally, we demonstrated that phenotypic changes appear in primary keratinocytes, similar to HaCaT cells, and the UVB-induced CPD- and ROS-dependent effects are keratinocyte-specific. To date, publications with regard to UVB have largely focused on DNA damage and carcinogenesis, ignoring metabolic changes, potential events that could lead to the emergence of the long-term effects of UVB. In addition, activation of signalling pathways between the nucleus and mitochondria are a completely uncharted area in photobiology. By modifying the efficiency of DNA repair, our results shed new light on the role of acute DNA damage following physiologically relevant doses of UVB irradiation, including the role of CPDs in the regulation of mitochondrial function. With such a unique methodological approach, our results are novel in photobiology; the introduction of the photolyase enzyme into the skin, the use of antioxidants, and the inhibition of certain metabolic pathways may have therapeutic potential in preventing UVB-induced effects.

6. PUBLICATION LIST

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List of publications related to the dissertation

1. **Hegedűs, C.**, Juhász, T., Fidrus, E., Janka, E. A., Juhász, G., Boros, G., Paragh, G. J., Uray, K., Emri, G., Remenyik, É., Bai, P.: Cyclobutane pyrimidine dimers from UVB exposure induce a hypermetabolic state in keratinocytes via mitochondrial oxidative stress.
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List of other publications

3. Fidrus, E., **Hegedűs, C.**, Janka, E. A., Paragh, G., Emri, G., Remenyik, É.: Inhibitors of Nucleotide Excision Repair Decrease UVB-Induced Mutagenesis - an In Vitro Study.
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