RESEARCH NOTE

Ultraviolet B, melanin and mitochondrial DNA: Photo-damage in human epidermal keratinocytes and melanocytes modulated by alpha-melanocyte-stimulating hormone [version 1; referees: 2 approved]

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Abstract

Alpha-melanocyte-stimulating hormone (alpha-MSH) increases melanogenesis and protects from UV-induced DNA damage. However, its effect on mitochondrial DNA (mtDNA) damage is unknown. We have addressed this issue in a pilot study using human epidermal keratinocytes and melanocytes incubated with alpha-MSH and irradiated with UVB. Real-time touchdown PCR was used to quantify total and deleted mtDNA. The deletion detected encompassed the common deletion but was more sensitive to detection. There were 4.4 times more mtDNA copies in keratinocytes than in melanocytes. Irradiation alone did not affect copy numbers. Alpha-MSH slightly increased copy numbers in both cell types in the absence of UVB and caused a similar small decrease in copy number with dose in both cell types. Deleted copies were nearly twice as frequent in keratinocytes as in melanocytes. Alpha-MSH reduced the frequency of deleted copies by half in keratinocytes but not in melanocytes. UVB dose dependently led to an increase in the deleted copy number in alpha-MSH-treated melanocytes. UVB irradiation had little effect on deleted copy number in alpha-MSH-treated keratinocytes. In summary, alpha-MSH enhances mtDNA damage in melanocytes presumably by increased melanogenesis, while α-MSH is protective in keratinocytes, the more so in the absence of irradiation.

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Introduction
Melanin is found in the principal cell cytoplasm as are the mitochondria. Although the principal role of melanin is photoprotection, the pigment is well known to emit melanin free radicals and act as a photosensitizing agent. These studies were undertaken to determine the effect of ultraviolet B irradiation (UVB) on the induction of reactive oxygen species damage of mitochondrial DNA (mtDNA) in cultures of pigment-induced human epidermal melanocytes compared to human epidermal keratinocytes. Deletions in mitochondrial DNA are the hallmark of reactive oxygen species damage.

Materials and methods
Cells and cell culture conditions
Normal human neonatal epidermal melanocytes were purchased from Tebu-bio (Portland, OR) (catalog # 104-05n 5E5 Cells) and normal human neonatal epidermal keratinocytes were obtained from PromoCell (Heidelberg, Germany) (catalog # C-12007). Melanocytes and keratinocytes were routinely cultured as reported previously. Melanocytes were maintained in MGM-M2 medium plus all supplements (Cascade Biologics, Portland, OR; M-254-500 plus S-500-2) while keratinocytes were cultured in KGM-2 medium with all supplements (PromoCell; C-20111). 500,000–700,000 cells were seeded into 6 cm diameter culture dishes. On the following day cells were deprived for 48 hrs from bovine pituitary extract followed by pre-incubation with 104 M α-MSH (α-melanocyte-stimulating hormone) (Calbiochem, Schwalbach, Germany) for 6 hrs at 37°C.

Irradiation
UVB treatment was performed with an irradiation bank consisting of six fluorescent bulbs (TL12, Philips) which emit most of their energy within the UVB range with an emission peak of 313 nm. Cells were irradiated through phosphate-buffered saline at 5, 10 and 15 ml/cm2 followed by incubation with experimental medium (5 ml) with and without α-MSH (104 M) for 24 hrs.

DNA extraction and purification
DNA was extracted using the Epicentre kit from Biozym (Hess. Oldendorf, Germany; MCD85201). DNA was finally dissolved in 10 mM Tris buffer with 0.1 mM EDTA.

Restriction and repurification
Five micrograms of each purified DNA sample were incubated with 10 units of AffIII (New England BioLabs, Ipswich, MA) for 1 hour at 37°C according to the manufacturer’s directions. The reaction mixture was repurified on spin columns according to the manufacturer’s directions (DNA Clean and Concentrator columns, Zymo Research Corporation, Orange, CA) and diluted appropriately in 10 mM Tris pH 8.5.

PCR
The Roche LightCycler-2 (Roche Applied Science, Indianapolis, IN) was used throughout these studies. Total mitochondrial genomes were quantified using primers HSSN1307 and HSAS1433 (5’ GTACCCAGCTAAAGACGTTAGG3’ and 5’TACTGCTAAATCCACC-TTGC3’ respectively) and probe 5’FAM-TGGCAGCCTAGCATTAGCAGTT-BHQ1-3’). Each capillary contained 250 pg DNA, 300 nM each primer, 200 nM probe, 0.01 μL uracil-DNAglycosylase, heat-labile (Roche) (UNG) and 1X LightCycler TaqMan Master Mix in a total volume of 5 μL. The Master Mix contains dUTP in place of dTTP in order for the amplified product to contain U in place of T. Pre-incubation of the PCR mixture with UNG allows for the degradation of any carryover contaminants from earlier reactions. The cycling protocol called for 10 min at 35°C for UNG digestion, followed by 10 min at 95°C to inactivate UNG and activate the Taq polymerase; up to 40 cycles of 95°C 10 sec for denaturation, 60°C 20 sec for annealing, 72°C 10 sec for polymerization and acquisition. Each run also contained two capillaries of 1 × 106 copies of the plasmid pKW into which the Total amplicon had been inserted.

Primer for the deletion were HSSN8416 and HSAS8542 (5’-CCCTACACTATTTCC-CATCACC-3’ and 5’-TGTGGTCTTTTGGAGTAGAAACC-3’ respectively). The probe was 5’-6FAM-TGGCAGCCTAGCATTAGCAGTT-BHQ1-3’). Each capillary contained 10 ng sample DNA, primers, probe, UNG and Master Mix as in the Total reactions. Glycosylation and denaturation were as for the Total PCR. Amplification was initiated with 5 cycles of Touchdown PCR: cycle 1: 95°C for 10 sec, 65°C for 5 sec, dropping 1 degree per cycle down to 60°C followed by no more than 55 cycles of 95°C for 10 sec, 60°C for 15 sec. Results with touchdown PCR were more reproducible and took fewer cycles to reach the crossing point. The touchdown amplicon was sequenced in the Molecular Resource Facility at the NJ Medical School.

The results shown are averages of three separate experiments. The proprietary software accompanying the Roche LightCycler was used to determine the crossing point of each sample. The crossing points of the samples were compared to crossing points of known copy numbers of standards in order to calculate sample copy numbers. In each experiment, total copies were determined in triplicate and deleted copies were determined in quintuplicate. Please see Dataset 1.

Results

Sequence of the touchdown amplicon
Gel analysis determined that the touchdown amplicon was smaller than that of the common deletion (CD): less than 100 bp versus 137 bp. These studies were undertaken to determine the effect of ultraviolet B irradiation (UVB) on the induction of reactive oxygen species damage of mitochondrial DNA (mtDNA) in cultures of pigment-induced human epidermal melanocytes compared to human epidermal keratinocytes. Deletions in mitochondrial DNA are the hallmark of reactive oxygen species damage.

Figure 1 shows the effect of UVB irradiation on total mitochondrial genome copy numbers in melanocytes and keratinocytes that have been treated with α-MSH, compared to untreated (no α-MSH) controls. In the absence of any α-MSH, keratinocytes have
4.4 times more mtDNA copies than melanocytes. Copy numbers of mtDNA from non-α-MSH treated cells of both cell types are unaffected by UVB exposure in this dose range. α-MSH causes a small increase in copy number in both cell types in the absence of irradiation and a parallel decline with dose.

Figure 2 shows the effect of UVB irradiation on deleted copy numbers in melanocytes and keratinocytes that have been exposed to α-MSH compared to non-α-MSH treated controls. In the absence of any α-MSH treatment, deleted copies are about half as frequent in melanocytes than in keratinocytes. In the absence of

**Figure 1.** Total mtDNA copies as a function of UVB dose. Black symbols: melanocytes, red symbols: keratinocytes. Circles: no α-MSH; squares: + α-MSH.

**Figure 2.** Deleted copies in melanocytes and keratinocytes as a function of UVB dose. Black symbols: melanocytes, red symbols: keratinocytes. Circles: no α-MSH; squares: + α-MSH.
α-MSH treatment, deleted copies decline slightly with increasing radiation dose in both types of cell. This is more likely due to slower replication of the deleted genomes than to their direct loss.

In melanocytes pre-treated with α-MSH, there is a marked increase in the frequency of deleted genomes with increasing UVB dose. This is likely to be due to the production of reactive oxygen species from UVB interaction with the melanin pigment or with melanin precursors. α-MSH actually causes a decrease in deleted copies in unexposed keratinocytes suggesting that, after exposure deleted copies replicate more slowly or that some protective agent has been induced. This effect is lessened as the UVB dose increases.

Discussion
The pigment melanin is widely distributed in the animal world and has multiple functions. In the skin, it is both photoprotective and photosensitizing. The quixotic nature of melanin has been the subject of a number of studies and reviews.

α-MSH is a key melanotropic factor induced by UVB irradiation in keratinocytes to turn on melanogenesis. Of note, we and others have shown that α-MSH is capable of reducing UVB-mediated DNA damage in both human melanocytes and keratinocytes independent from its melanin-inducing effect. Moreover, we recently found that α-MSH has indirect anti-oxidative effects on human melanocytes and keratinocytes since it upregulates expression of the transcription factor Nrf2, a master regulator of phase II detoxifying enzymes. Thus, we speculated that in both cell types α-MSH may also protect from some UVB-induced oxidative effects on mtDNA. The CD in mtDNA is a hallmark for oxidative stress. Arck et al. reported more CDs in graying hair follicles than in unpigmented follicles, suggesting that the pigment was responsible for the effect. Our findings in fact suggest that α-MSH-induced melanin synthesis acts as a photosensitizer for mtDNA damage in the form of the TD after irradiation with UVB in the MED (minimal erythema dose) range. The presence of melanin and/or its precursors in the cytoplasm may indeed produce oxidative damage in mtDNA which is enhanced by solar irradiation, thus altering the "milieu intérieur" and possibly leading to altered mitochondrial function. In this context, it should be very interesting to determine the direct influence of α-MSH alone or in combination with UVB on mitochondrial metabolism and biogenesis. In addition, investigation on the impact of signaling-deficient MC1R alleles on mtDNA damage in melanocytes treated with α-MSH and UVB may disclose unexpected clues in the pathogenesis of cutaneous melanoma.

Recently, Premi et al. irradiated mouse and human pigmented and non-pigmented cells with UVA and measured the production of cyclobutane pyrimidine dimers (CPDs) in total cellular DNA. They found that CPDs continued to increase in the pigmented cells, but not in the non-pigmented cells, for as long as 3 hours after irradiation, after which they declined due to repair. In our study, deletions were measured at only a single time point so there is no way of determining the dynamics of formation. However, once formed, they would persist unrepaired. It would be of interest to study the time-dependent effects of UVB and UVA on induction of mitochondrial deletions in unpigmented, pigmenting and pigmented cells, more especially since Boulton and Birch-Machin recently found that pigment is protective against reactive oxygen generation by complex II. Another demonstration, perhaps, of the dual nature of melanin as a two-edged sword. Function is protected while genetic information is damaged.

Conclusion
There are about 2.4 times more deleted copies relative to total copies in melanocytes compared to keratinocytes. UVB increases the ratio in the presence of α-MSH in melanocytes while it remains relatively stable in keratinocytes. This suggests that α-MSH causes a destabilization of the free-radical balance in melanocytes but not in keratinocytes. This effect may be the result of the increase in melanin precursors and/or pigment by the presence of α-MSH.

Data availability
F1000Research: Dataset 1. Raw data for ‘Ultraviolet B, melanin and mitochondrial DNA: Photo-damage in human epidermal keratinocytes and melanocytes modulated by alpha-melanocyte-stimulating hormone’, Böhm and Hill 2016, 10.5256/f1000research.8582.d121454.

Author contributions
MB and HZH together conceived the study. MB directed the cell culture, irradiations, cell harvesting and DNA extractions. HZH restricted, repurified the DNA and performed the Real Time PCR. HZH and MB contributed equally to the manuscript preparation and agree to the final content.

Competing interests
No competing interests were disclosed.

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References

1. Collins B, Poehler TO, Bryden WA: EPR persistence measurements of UV-induced melanin free radicals in whole skin. Photochem Photobiol. 1995; 62(3): 557–560. PubMed Abstract | Publisher Full Text

2. Meredith P, Sama T: The physical and chemical properties of eumelanin. Pigment Cell Res. 2006; 19(6): 572–594. PubMed Abstract | Publisher Full Text

3. Kotha A, Metze D, Mouchet N, et al.: Alpha-melanocyte-stimulating hormone counteracts the suppressive effect of UVB on H2O2 and H2O-dependent gene expression in human skin. Endocrinology; 2009; 150(7): 3197–3206. PubMed Abstract | Publisher Full Text | Free Full Text

4. Pogozelski WK, Hamel CJ, Woeller CF, et al.: 2-carboxylic acid enhances oxidative damage in human keratinocyte DNA damage and UV-induced photoproducts. Photochem Photobiol. 2001; 74(3): 415–427. PubMed Abstract | Publisher Full Text | Free Full Text

5. Don RH, Cox PT, Wainwright BJ, et al.: ‘Touchdown’ PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. 1991; 19(14): 4008. PubMed Abstract | Publisher Full Text | Free Full Text

6. Bykov VJ, Marcusson JA, Hemminki K: Effect of constitutional pigmentation on total mitochondrial DNA and the 4977-bp common deletion in Pearson’s syndrome lymphoblasts. J Invest Dermatol. 2000; 114(1): 40–43. PubMed Abstract | Publisher Full Text | Free Full Text

7. De Leeuw SM, Smit NP, Van Veldhoven M, et al.: Melanin content of cultured human melanocytes and UV-induced cytotoxicity. J Photochem Photobiol B. 2001; 61(3): 106–113. PubMed Abstract | Publisher Full Text | Free Full Text

8. Smit NP, Vink AA, Kob RM, et al.: Melanin offers protection against induction of cyclobutane pyrimidine dimers and 6-4 photoproducts by UVB in cultured human melanocytes. Photochem Photobiol. 2001; 74(3): 424–430. PubMed Abstract | Publisher Full Text | Free Full Text

9. Kvw E, Tyrell RM: Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation. Carcinogenesis. 1997; 18(12): 2379–2384. PubMed Abstract | Publisher Full Text

10. Marrot L, Beladi JP, Meunier JR, et al.: The human melanocyte as a particular target for UVA radiation and an endpoint for photoprotection assessment. Photochem Photobiol. 1999; 69(6): 686–693. PubMed Abstract | Publisher Full Text | Free Full Text

11. Takeuchi S, Zhang W, Wakamatsu K, et al.: Melanin acts as a potent UVB photosensitizer to cause an atypical mode of cell death in murine skin. Proc Natl Acad Sci USA. 2004; 101(42): 15076–15081. PubMed Abstract | Publisher Full Text | Free Full Text | Publisher Full Text

12. Wang HT, Choi B, Tang MS: Melanocytes are deficient in repair of oxidative DNA damage and UV-induced photoproducts. Proc Natl Acad Sci USA. 2010; 107(27): 12180–12185. PubMed Abstract | Publisher Full Text | Free Full Text

13. Kpp C, Young AF: The soluble eumelanin precursor 5,6-dihydroxyindole-2-carboxylic acid enhances oxidative damage in human keratinocyte DNA after UVA irradiation. Photochem Photobiol. 1999; 70(2): 191–198. PubMed Abstract | Publisher Full Text

14. Hill HZ: The function of melanin or six blind people examine an elephant. Biosciences. 1992; 14(1): 49–56. PubMed Abstract | Publisher Full Text | Free Full Text

15. Hill HZ, Li W, Xin P, et al.: Melanin: a two edged sword? Pigment Cell Res. 1997; 10(3): 158–161. PubMed Abstract | Publisher Full Text

16. Hill HZ, Hill GJ, Cieszka K, et al.: Comparative action spectrum for ultraviolet light killing of mouse melanocytes from different genetic coat color backgrounds. Photochem Photobiol. 1997; 65(6): 983–989. PubMed Abstract | Publisher Full Text

17. Hill HZ, Hill GJ: UVA, pheomelanin and the carcinogenesis of melanoma. Pigment Cell Res. 2000; 13(Suppl 8): 140–144. PubMed Abstract | Publisher Full Text

18. Goodman G, Berovoch D: Melanin directly converts light for vertebrate metabolic use: heuristic thoughts on birds, icarus and dark human skin. Med Hypotheses. 2006; 71(2): 190–202. PubMed Abstract | Publisher Full Text

19. Böhm M, Wolff I, Schönzen TE, et al.: alpha-Melanocyte-stimulating hormone protects from ultraviolet radiation-induced apoptosis and DNA damage. J Biol Chem. 2005; 280(7): 5795–5802. PubMed Abstract | Publisher Full Text

20. Kadekaro AL, Kavanagh R, Kanto H, et al.: alpha-Melanocortin and endothelin-1 activate antiapoptotic pathways and reduce DNA damage in human melanocytes. Cancer Res. 2005; 65(19): 4292–4299. PubMed Abstract | Publisher Full Text

21. Dong L, Wen J, Pier E, et al.: Melanocyte-stimulating hormone directly enhances UV-induced DNA repair in keratinocytes by a xeroderma pigmentosum group A-dependent mechanism. Cancer Res. 2010; 70(9): 3547–3556. PubMed Abstract | Publisher Full Text | Free Full Text

22. Arik PC, Overall R, Spatz K, et al.: Towards a “free radical theory of graying”: melanocyte apoptosis in the aging human hair follicle is an indicator of oxidative stress induced tissue damage. FASEB J. 2006; 20(9): 1567–1569. PubMed Abstract | Publisher Full Text

23. Premi S, Wallisch S, Mano CM, et al.: Photochemistry. Chemiexcitation of melanin derivatives induces DNA photoproducts long after UV exposure. Science. 2015; 347(6224): 842–847. PubMed Abstract | Publisher Full Text | Free Full Text

24. Boulton SJ, Birch-Machin MA: Impact of hyperpigmentation on superoxide flux and melanoma cell metabolism at mitochondrial complex II. FASEB J. 2015; 29(1): 346–53. PubMed Abstract | Publisher Full Text

25. Böhm M, Hill HZ: Dataset 1 in: Ultraviolet B, melanin and mitochondrial DNA: Photo-damage in human epidermal keratinocytes and melanocytes modulated by alpha-melanocyte-stimulating hormone. F1000Research. 2016. Data Source
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This report from Markus Böhm and Helene Hill describes a study on the mitochondrial DNA damaging effect of ultraviolet B (UVB) radiation in keratinocytes and melanocytes, in the presence or absence of the melanotropic peptide hormone alphaMSH. It is known that binding of this melanocortin to its cognate receptor expressed in melanocytes, the MC1R, triggers a series of pleiotropic responses notably including activation of the melanogenic pathway and induction of antioxidant enzymes as well as DNA repair pathways. It is also known that MC1R is expressed in many nonmelanocytic cell types including keratinocytes. In fact, work in M Böhm’s lab has been crucial to the current view of alphaMSH/MC1R signaling as a key photoprotective process, and has led the field of extramelanocytic actions of MC1R. The contributions of HZ Hill have been also important to appreciate the dual character of melamins, with photoprotective roles but also endowed with a photosensitizing potential. Accordingly, the work is presented by leading experts in the field.

Whereas the role of alphaMSH in mediating protection of nuclear DNA against UVB is well established, not much is known about a possible action on mitochondrial DNA. Therefore, the paper has the merit to address a still unexplored question. The authors report a higher frequency of damaged mitochondrial DNA in control keratinocytes not exposed to UVB, compared with control melanocytes. Rather counterintuitively, alphaMSH apparently protects mitochondrial DNA against UVB-induced damage in keratinocytes, whereas it promotes an increase in the number of damaged copies in melanocytes. Since the hormone stimulates melanogenesis in melanocytes but not in keratinocytes, this suggests that the melanin polymer or some unidentified melanogenic intermediates might in fact act as photosensitizers, in keeping with the two-edged sword character of melanin emphasized by previous contributions from HZ Hill.

The experiments reported here are well described and most likely well conducted, and I have not detected any overinterpretation of the results. One caveat that leaves room for improvement is the absence of a statistical analysis of the small differences between the different experimental conditions.

A major strength of this short report is that it raises new questions that are likely to be addressed in the near future. Do eumelanin-containing melanocytes from dark-skinned donors display the same responses to UVB in terms of mitochondrial DNA damage as feomelanogenic melanocytes from individuals with red hair color? Do melanocytes expressing the consensus MC1R respond to alphaMSH comparably to melanocytes expressing variant MC1R? Is there a differential effect of alphaMSH in melanocytes challenged with UVB in the presence of inhibitors of the rate-limiting melanogenic enzyme tyrosinase, or...
for that matter in albino melanocytes? And what is the impact of UVB and alphaMSH signaling on the performance of oxidative phosphorylation/electron transport chain as a main source of DNA-damaging reactive oxygen species? Some of these questions are mentioned in the short but well focused discussion, and their answer will contribute to a better understanding of the complex role of pigment production in the homeostasis of UVB-irradiated human skin.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

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This is well performed study by experts in the field. The methodology and data collection are sound and data properly discussed.

I believe that that careful readers would like to have more extended list of references plus appropriate comments in the introduction and discussion.

For example on the capability of the skin to express POMC and its processing to MSH, ACTH and endorphin peptides, on hormonal and nutritional regulation of melanin pigmentation with regulatory consequences, and double-edge sword role of melanin in melanoma.

References
1. Slominski A, Wortman J, Luger T, Paus R, Solomon S: Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev. 2000; 80 (3): 979-1020 PubMed Abstract
2. Slominski A, Tobin DJ, Shibahara S, Wortman J: Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. 2004; 84 (4): 1155-228 PubMed Abstract I Publisher Full Text
3. Slominski A, Zmijewski MA, Pawelek J: L-tyrosine and L-dihydroxyphenylalanine as hormone-like regulators of melanocyte functions. Pigment Cell Melanoma Res. 2012; 25 (1): 14-27 PubMed Abstract I Publisher Full Text
4. Slominski RM, Zmijewski MA, Slominski AT: The role of melanin pigment in melanoma. Exp Dermatol. 2015; 24 (4): 258-9 PubMed Abstract I Publisher Full Text

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.