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Title	Blue light ($\lambda=453$ nm) nitric oxide dependently induces β -endorphin production of human skin keratinocytes in-vitro and increases systemic β -endorphin levels in humans in-vivo
Article type	Original article

Abstract

β -Endorphin exerts a broad spectrum of physiological activity on mood, immune functions, pain management, reward effects, and behavioral stability. β -Endorphin is produced in certain neurons within the central and peripheral nervous system but also in the skin, especially in response to ultraviolet radiation. In the present study we have investigated the impact of visible blue light at $\lambda=453$ nm (BL) on β -endorphin production of primary human skin keratinocytes (hKC) in-vitro as well as on systemic β -endorphin formation of whole-body exposed subjects in-vivo. We found that BL irradiation significantly enhanced both keratinocytic β -endorphin production of hKC cultures as well as systemic β -endorphin concentrations in light exposed healthy subjects. Interestingly, in hKC cultures elevated β -endorphin formation was paralleled by significantly increased levels of non-enzymatically generated nitric oxide (NO), whereas elevated systemic β -endorphin values of BL-exposed subjects were accompanied by enhanced systemic concentration of bioactive NO-derivates. These findings point to a pivotal role of NO in the molecular mechanism of the observed BL-induced effects, and indeed, exogenously applied NO was able to significantly enhance β -endorphin production in hKC cultures. Thus, our finding of BL-induced increases in systemic β -endorphin concentration in-vivo can be plausibly explained by an event sequence comprising 1.) BL-driven non-enzymatic formation of NO in the exposed skin tissue, 2.) systemic distribution of cutaneously produced NO, 3.) a subsequent NO-dependent induction of β -endorphin synthesis in epidermal keratinocytes, and 4.) probably also a NO-dependent modulation of β -endorphin synthesis in specialized neurons within the central and peripheral nervous system.

Keywords	Nitric oxide; beta-endorphin; blue light; keratinocytes, POMC; in vivo study
Taxonomy	Endocrine System Physiology, Molecular Biology Experimental Approach, Human Physiology, Molecular Medicine
Corresponding Author	Christoph Suschek
Corresponding Author's Institution	University Hospital of the Heinrich-Heine-University Düsseldorf
Order of Authors	Isabel Albers, Erika Zernickel, Manuel Stern, Melanie Broja, Lucas Busch, Christian Heiss, Vera Grotheer, Joachim Windolf, Christoph Suschek
Suggested reviewers	Erna Snellman, Martin Feelisch, Richard Weller, David E Fisher, Hsin-Chi Tsai

Submission Files Included in this PDF

File Name [File Type]

Cover Letter 2.pdf [Cover Letter]

Point-by-Point Reply.pdf [Response to Reviewers]

Highlights.pdf [Highlights]

Graphical Abstract.pdf [Graphical Abstract]

Albers et al.pdf [Manuscript File]

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Dear Editor,

We have edited the reviewer's and editor's comments point by point, and hopefully answered all the questions posed satisfactorily. Additionally, all desired corrections and additions of the manuscript have been fulfilled in full.

We hope that revising the manuscript and answering the questions will make it easier for you and the reviewers to accept our manuscript for publication in ***Free Radical Biology and Medicine***.

Yours sincerely,

A handwritten signature in black ink, appearing to read 'Christoph V. Suschek', written in a cursive style.

Christoph V. Suschek. Ph.D.

Trauma Research Group
Department of Trauma and Hand Surgery
University Clinics of the Heinrich-Heine-University Düsseldorf
Merowinger Platz 1A
40225 Düsseldorf
Germany

Phone: +49 (0)211-302039-234

E-mail: suschek@hhu.de

Point-by-Point Reply

Comments from the reviewers

The only comment the reviewer made was the manuscript required revisions in the discussion section to improve clarity and better explain the results.

Author's reply

We have thoroughly revised the discussion section and think that it is much more understandable now. We underlined changed text passages.

Comments from the editors

Comment 1

Please clarify if sulfanilamide in acid reacts with nitrite was used in the quantification of NO metabolites in serum and in cells.

Author's reply

We now clarify this fact in the MM-section, page 8, last paragraph. Text changes are underlined: " Nitrite concentrations were calculated by the difference in peak areas of untreated aliquots and of those subjected to pre-incubation with 0.5% sulfanilamide/HCl, the latter representing total nitrosated species".

Comment 2

Please clarify if and how small molecular weight SNO adducts (i.e. S-nitrosogluthathione) were distinguished from protein S-nitrosocysteine. This is important regarding the possible mechanistic interpretation of the data.

Author's reply

As part of your commentary, we noticed that we were sometimes very misleading. In the blood samples we have determined only the sum of all nitroso compounds (RX-NO). We have not differentiated between S- and N-nitroso compounds. We did not consider the distinction to be necessary because we assumed that the concentration of RX-NO strictly correlates with the concentration of the more relevant RS-NO species.

In the keratinocyte homogenates, on the other hand, we quantified the concentration of all S-nitroso compounds. We have not distinguished between small and large molecular S-nitrosospecies. However, there is no disadvantage here, since blue light cleaves small and large molecular weight S-nitrosospecies to the same extent with NO release.

The respective text changes in the MM-section are underlined (page 8. Last paragraph; page 10, second paragraph).

Comment 3

Please indicate the negative controls that tested the specificity of the antibodies. The use of NOS inhibitor and/or pre-absorption must be used to secure specificity.

Author's reply

To detect S-nitrosation in keratinocytes, we used an S-nitrosoprotein-specific antiserum, since corresponding monoclonal antibodies can not be purchased commercially. We routinely check the reliability of the antiserum on nitrosated albumin samples by Western blot. As a negative control, we have incubated the corresponding samples only with the second antibody, i.e. without the application of the first antibody. We now mention these points at the appropriate place in the MM section (page 15, second paragraph, underlined text).

We absolutely agree with the second point of Comment 3. In order to evaluate the impact of non-enzymatic NO it is necessary to exclude possible effects of enzymatically produced NO. We therefore have irradiated keratinocyte cultures with blue light (453 nm, 80 J/cm²) in the presence of 1.0 mM L-NIO, an effective NO-Synthase inhibitor. We mention this fact in the legend of figure 3 but unfortunately not in the text of the MM-section. We have done this now (page 14, second paragraph, underlined text).

In the literature L-NIO is described as an iNOS-specific NOS-inhibitor. Nevertheless, at the used concentration of 1 mM L-NIO also completely inhibits eNOS as well nNOS activity.

Comment 4

DAF-FM does not react with NO. It reacts with a mire reactive higher oxides derived from NO. Please see the article below and revise accordingly. Kalyanaraman B, Darley-Usmar V, Davies KJ, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ 2nd, Ischiropoulos H. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic Biol Med. 2012 52(1):1-6.PubMed PMID: 22027063; PubMed Central PMCID: PMC3911769].

Author's reply

Thanks for the hint. We have now corrected the incorrect information (page 14, first paragraph).

Comment 5

According to the working hypothesis blue light increases release of NO from keratinocytes. The data indicates that exposure to light increases NO-adducts within keratinocytes (the pre- and post light exposure is rather remarkable) first before a subsequence release. However, it is unclear at which stage this relates to β -endorphin production and release. There is an association between the two but not a clear cause and effect.

Author's reply

The questions in Comment 5 bare the weaknesses of the study. We can only indirectly substantiate our claim of blue light-induced endorphin synthesis: 1.) we see that blue light induces nonenzymatic generation of NO in keratinocytes which strongly correlates with keratinocytic endorphin synthesis, and 2.) that exogenously applied NO can increase endorphin syn-

thesis in keratinocytes. From this we conclude that NO generated by blue light also induces endorphin synthesis in keratinocytes.

Methodologically, this is a well-established and accepted evidence chain, and our conclusions are fully valid, although we can not provide any direct evidence that intracellularly-produced NO is also capable of keratinocytic endorphin synthesis. We tried to solve this problem by using the NO-specific scavenger cPTIO during light exposure. Unfortunately, we found that cPTIO decays due to the influence of blue light and therefore can not be used to solve our problem

Comment 6

Why and what is released in circulation. If it is NO then the increase in SNO adducts requires a secondary chemistry and/or catalysis to form SNO adducts.

Author's reply

As we mention in the discussion, irradiation of the skin with light sources capable of cleaving photolabile NO derivatives (e.g. UVA radiation at 375 nm or blue light at 453 nm) results in a strong increase in non-enzymatically generated NO in the skin as well as NO-specific biological responses. In the case of a local light exposure, we observed erythema formation as well as increased local blood flow. In the case of a full-body exposure, however, we also observed systemic effects on hemodynamics. The systemic hemodynamic effects after light exposure correlate with an increase in serum nitrite as well as RS-NO concentration. These nitrosated NO equivalents mediate the biological effects of NO on local and systemic hemodynamics. We therefore assume that RX-NO is also responsible for the NO-induced endorphin synthesis observed in rats, and that in our experiments they are the actual bioactive agent. We mention these facts in the Discussion section. (page 21, first paragraph)

Comment 7

What is the biological function of the NO/SNO released by light. The discussion is unclear. Does it increase local blood flow or systemic blood flow. Does the increased blood flow required for the biological function of β -endorphin?

Author's reply

Our group was the first to show that human skin is a large reservoir of photolabile NO derivatives that can decay to NO under the influence of light of a specific wavelength and induce biological effects. Several groups are now working in this area and it is agreed that this system of non-enzymatic light-induced NO generation might make a not insignificant contribution to the regulation of blood pressure. A keyword to be mentioned in this context would be e.g. the phenomenon of the so-called French Paradox. Against this background, it would not be surprising if light or sunlight could, via the same mechanism, also affect other factors of human physiology, e.g. the psyche by regulating the expression of corresponding psychogenic factors.

Concerning the second question please see author's reply to Comment 6.

Concerning the last question in comment 7 it was possible to show that hypertensive patients showed higher beta-endorphin plasma levels than normotensive subjects ($p < 0.002$). On the other side it was observed that high doses of β -endorphin induce hypotensive and beneficial

hormonal effects in humans, which are enhanced in essential hypertension and are mediated by opioid receptors.

Highlights

In addition to its essential role in vitamin D synthesis, the environmental factor sunlight, especially its ultraviolet (UV) fraction, also strongly affects the human psyche, e.g. by modulating the synthesis psychoactive mediators like β -endorphin. Here we show for the first time with human skin keratinocyte cultures but also with whole-body-irradiated healthy volunteers that visible UV radiation-free blue light, which has a much lower risk for side effects than UV radiation, also can induce a significant increase in local as well as systemic production of β -endorphin. The underlying mechanism comprises an enhanced local or systemic generation of nitric oxide (NO) via blue light-induced decomposition of photo-labile NO derivatives. Thus, based on our findings, blue light might have a relevant clinical potential to serve as supportive measure in the therapy of several psychiatric disorders that might be modulated by β -endorphin.

453 nm

step 1

step 2a

step 2b

skin
keratinocytes

photodecomposition of
photolabile NO-derivates

local effects of NO

local effects of $\beta\text{-End}$

systemic distribution of
 $\beta\text{-End}$

systemic effects of $\beta\text{-End}$

systemic effects of NO

local effects of NO

systemic distribution of NO

systemic effects of NO:

e.g. blood pressure ↓

$\beta\text{-End}$ synthesis ↑

Blue light ($\lambda=453$ nm) nitric oxide dependently induces β -endorphin production of human skin keratinocytes *in-vitro* and increases systemic β -endorphin levels in humans *in-vivo*

Isabel Albers ¹, Erika Zernickel ¹, Manuel Stern ², Melanie Broja ², Lukas Busch ², Christian Heiss ^{3,4}, Vera Grotheer ¹, Joachim Windolf ¹, Christoph V. Suschek ^{1, #}

¹ Department of Trauma and Hand Surgery, Medical Faculty of the Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

² Division of Cardiology, Pulmonology, and Vascular Medicine, Heinrich-Heine University, Medical Faculty, University Düsseldorf, Germany

³ University of Surrey, Faculty of Health and Medical Science, Guildford, United Kingdom

⁴ Surrey and Sussex NHS Healthcare Trust, Redhill, United Kingdom

Corresponding author:

Prof. Dr. Christoph V. Suschek

Department of Trauma and Hand Surgery

Heinrich Heine University Hospital Düsseldorf

Moorenstr. 5

40225 Düsseldorf

Germany

E-mail: suschek@uni-duesseldorf.de

SUMMARY

β -Endorphin exerts a broad spectrum of physiological activity on mood, immune functions, pain management, reward effects, and behavioral stability. β -Endorphin is produced in certain neurons within the central and peripheral nervous system but also in the skin, especially in response to ultraviolet radiation. In the present study we have investigated the impact of visible blue light at $\lambda=453$ nm (BL) on β -endorphin production of primary human skin keratinocytes (hKC) *in-vitro* as well as on systemic β -endorphin formation of whole-body exposed subjects *in-vivo*. We found that BL irradiation significantly enhanced both keratinocytic β -endorphin production of hKC cultures as well as systemic β -endorphin concentrations in light exposed healthy subjects. Interestingly, in hKC cultures elevated β -endorphin formation was paralleled by significantly increased levels of non-enzymatically generated nitric oxide (NO), whereas elevated systemic β -endorphin values of BL-exposed subjects were accompanied by enhanced systemic concentration of bioactive NO-derivates. These findings point to a pivotal role of NO in the molecular mechanism of the observed BL-induced effects, and indeed, exogenously applied NO was able to significantly enhance β -endorphin production in hKC cultures. Thus, our finding of BL-induced increases in systemic β -endorphin concentration *in-vivo* can be plausibly explained by an event sequence comprising 1.) BL-driven non-enzymatic formation of NO in the exposed skin tissue, 2.) systemic distribution of cutaneously produced NO, 3.) a subsequent NO-dependent induction of β -endorphin synthesis in epidermal keratinocytes, and 4.) probably also a NO-dependent modulation of β -endorphin synthesis in specialized neurons within the central and peripheral nervous system.

Key words: Nitric oxide, beta-endorphin, blue light, keratinocytes, POMC, in vivo study

INTRODUCTION

β -endorphin is an endogenous opioid neuropeptide and hormone that is produced in certain neurons within the central and peripheral nervous system (for review see [1, 2]). β -endorphin exerts local as well as global functions. Global function of this neuropeptide comprises pain management, reward effects, behavioral stability, stress response, immune regulation, and decreasing bodily stress [1-3]. β -endorphin binds with high affinity to the presynaptic μ -opioid receptor, where morphine and heroine bind as well and it exerts pronounced analgesic property [2], which is up to 100 times the analgesic potency of morphine [2]. β -endorphin modulates pain perception both in the central nervous system and the peripheral nervous system [3].

In humans β -endorphin is derived from its precursor protein pro-opiomelanocortin (POMC), which is predominantly synthesized in the pituitary gland, but also in the central nervous system and in several peripheral tissues (for review see [2]). POMC is posttranslationally modified by prohormone convertase 1/3 (PC1/3) and PC2. PC1/3 cleaves POMC into adrenocorticotrophic hormone (ACTH) and β -lipotropin (β -LPH). Subsequently PC2 processes β -LPH to γ -LPH and the C-terminal fragment β -endorphin.

In addition to tissues of the nervous system, also in the skin a part of the neuroendocrine system could be detected in keratinocytes and melanocytes [2, 4]. In human skin keratinocytes ultraviolet (UV) B-radiation was shown to induce a pronounced POMC expression by a mechanism comprising activation of the tumor suppressor gene p53 [5]. Keratinocytic prohormone convertases can cleave POMC up to β -endorphin that was shown to be secreted in significant amounts in keratinocyte cell culture *in-vitro* after UVB-exposure [2, 6]. In rodents a systemic UVB-treatment *in-vivo* was shown to lead to POMC-derived β -endorphin synthesis in the skin and resulted in significantly enhanced plasma levels of β -endorphin and α -MSH [7, 8]. Interestingly, UVB exposure of mice was shown to lead in a signaling system in the arcuate nucleus and the expression of the POMC gene, an

observation suggesting that exposure of skin to UVB radiation has a profound far-reaching influence on brain activity [8].

As part of our research on the physiological role of nitric oxide (NO) during exposure of human skin to energy-rich radiation we recently could observe that UVA-radiation [9] but also blue light at 453 nm [10] were able to initiate a significant non-enzymatic formation of NO in the irradiated skin area. This NO was the product of photodecomposition of photolabile NO derivatives. In the case of whole body exposure of healthy volunteers to UVA or blue light the enhanced cutaneous formation of non-enzymatic NO resulted in significantly augmented systemic concentrations of bioactive NO derivatives as well as in markedly increased systemic NO-mediated physiological effects, i. e. reduced blood pressure [9, 11].

The last point concerning the systemic effects of NO is of particular importance as there are many hints within the existing literature that NO might be directly or indirectly involved in the activation of the pituitary pro-opiomelanocortin system leading to elevated formation of POMC-derived peptides in the central nervous system or peripheral tissues. In this context it could be demonstrated that nitrous oxide (N₂O) induces neuronal release of β -endorphin formation in a strong NO-dependent manner [12, 13], that at pituitary level NO is involved in basal and interleukin-1 β -induced ACTH release *in-vitro* [14], and that N-methyl-D-aspartate (NMDA)-stimulated release of α MSH from the rat hypothalamus occurs via release of nitric oxide [15]. Furthermore, in mice lacking the neuronal NO synthase (nNOS) EP-immunoreactivity in the arcuate nucleus [16] and the expression of POMC and POMC-derived peptides in the mouse pituitary was reduced [17]. Finally, Hiramoto *et al.* could demonstrate that UVA-irradiation of the eye can activate a nitric oxide-dependent hypothalamo-pituitary pro-opiomelanocortin pathway [18].

Against this background we here have investigated the impact of blue light on β -endorphin production of primary human skin keratinocytes *in-vitro* as well as on β -endorphin values of blood samples of whole-body blue light-exposed subjects *in-vivo*.

MATERIALS AND METHODS

Materials

If not otherwise indicated, all chemicals, antibodies, and assay kits were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany).

LED-Arrays

For the *in-vitro* experiments we used a narrow-band LED-device (12 x 10 cm LED-arrays with 60 LEDs) emitting monochromatic light with a maximum intensity at 453 ± 10 nm (blue). LED-arrays were provided by Philips Research (Aachen, Germany). Prior delivery the irradiance of the LED-device was characterized using an integrating (Ulbricht) sphere. If not otherwise indicated, the device was used at an irradiance of 38 mW/cm^2 at a distance of 8 cm.

The device used in the *in-vivo* study was a full body blue light device from Philips Light & Health (Eindhoven, The Netherlands). The device was a non-CE marked prototype but it has been built according to the basic electrical safety standard for medical devices IEC 60601-1. It was equipped with 720 LEDs emitting UV-free blue light with a peak wavelength of about 450 ± 5 nm. The irradiance level was about 40 mW/cm^2 at a distance of 50 cm from the skin resulting in a fluence of 72 J/cm^2 within 30 minutes.

Clinical pilot study

A.) Study design, endpoints, and protocol

This study was designed as a 2 arm randomized, controlled, cross over study, exactly as shown recently [11]. The 14 healthy male volunteers were exposed in random order to monochromatic blue light (450 nm) and control light (filter foil covering the volunteers) each on a different day separated by one week of wash-out delivered by the same irradiation device. The primary endpoint of the study was a significant change in peripheral blood

pressure after 30 minutes of blue light (450 nm) irradiation compared to the control irradiation. Secondary endpoints included concentrations of β -endorphin and nitroso-compounds (RX-NO) in plasma and changes in several systemic as well as local hemodynamic parameters.

After placement of an intravenous catheter in the left cubital vein, volunteers rested over 30 minutes in a supine position on the irradiation device in a quite air conditioned room only wearing swimming shorts and safety glasses for acclimatization. Baseline measurements were taken immediately before exposure to 30 min irradiation of blue light or control light and were followed over 2 hours. For control light exposure, the volunteers were covered during irradiation with a thin opaque foil. Average skin temperature increase during blue light irradiation was comparable to the temperature increase below the foil used for control irradiations ($\Delta T = 7 \pm 1 \text{ }^{\circ}\text{C}$). Blood draws for analysis of circulating β -endorphin and nitric oxide derivatives were drawn via an intravenous catheter at baseline (-60 min), immediately after irradiation (0 min) as well as 60 and 120 minutes after light exposure. The study protocol was approved by the *Ethics Committee of the Heinrich Heine University Duesseldorf* and all volunteers gave written informed consent (Clinicaltrials.gov: NCT03226587).

B.) Study subjects

Fourteen healthy Caucasian men, between 30-60 years of age were recruited at the University of Düsseldorf (August 2016-August 2017). The study subjects were screened based on a clinical physical exam including blood pressure measurement, EKG, and routine clinical test (blood lipids, CRP, full blood count, liver enzymes, hemoglobin, glucose). Inclusion criteria for participation in the study were an age of 30-60 years, Caucasian skin type, male gender, and signed consent form. Exclusion criteria were diabetes mellitus, acute inflammation, arrhythmia, active malignancy, terminal renal failure, manifest cardiovascular

disease, i.e. coronary artery disease, peripheral artery disease, cerebrovascular disease, heart rhythm other than sinus, arterial hypotension (systolic <100 mmHg), active medical treatment with blood pressure lowering medication, photodermatosis and/or photosensitivity, porphyria and/or hypersensitivity to porphyrins, congenital or acquired immunodeficiency, subjects with genetic deficiencies attached with increased sensitivity to light or increased risk to dermatologic cancer (i.e. Xeroderma pigmentosum, Cockayne Syndrome, Bloom-Syndrome).

Blue light-induced emanation of nitric oxide from human skin *in-vivo*

Blue light at 453 nm is able to generate intracutan nitric oxide (NO) formation as a result of photodecomposition of photolabile NO-derivates in human skin [10]. In order to quantify NO emanation from human skin during blue-light exposure, we used a stainless steel collection chamber for the accumulation of emanated gases from human skin. The cylindrical airtight chamber covered a skin area of 16.0 cm², had a height of 2.2 cm and a total volume of 35.2 cm³ [19]. The light transparent front window allowed illumination of the underlying skin. The chamber was placed on the clean inner forearm of volunteers and flushed with an inert gas (nitrogen or helium) for 1 min to remove oxygen [20]. After stopping the gas flow, NO from untreated as well as irradiated skin (see LED light sources mentioned above) was accumulated in the closed chamber for 180 s. The NO content of the chamber was quantified by flushing the contents with the trace gas into the chemiluminescence-based NO detector CLD 88e from Eco Physics (Duernten, Switzerland).

Quantification of nitrite and nitroso compounds (RX-NO) in blood plasma by CLD

The concentrations of nitrite and nitrosated compounds (RX-NO), sum of N- and S-nitroso-compounds, which represent the transport form of nitric oxide in blood, were quantified in serum samples obtained from blue light (450 nm)-irradiated as well as sham treated subjects

of the study by reductive denitrosation using a mixture of iodine/iodide in glacial acetic acid and subsequent detection of the liberated NO by its gas-phase chemiluminescence reaction with ozone, essentially as described previously [21, 22]. Nitrite concentrations were calculated by the difference in peak areas of untreated aliquots and of those subjected to preincubation with 0.5% sulfanilamide/HCl, the latter representing total nitrosated species [23]. The chemiluminescence detector was calibrated weekly using a 100 ppb mixture of NO in N₂ or He₂ and calculated NO amounts were validated by injection of freshly prepared nitrite standards into the reaction mixture.

Establishment of primary skin keratinocyte cell cultures

Human skin specimens were obtained with patients' consent from twelve male and female patients aged between 20 and 65 who underwent plastic breast or abdominal surgery. The experimental protocol and the use of human material has been approved by the local *Ethics Committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf* (study number: 3634). After rinsing skin specimens in phosphate buffered saline (PBS) subcutaneous fat was removed, the skin was sliced in pieces (1 x 0.5 cm) and incubated in 0.1% Dispase grade II (Roche) at 4 °C, overnight. The next day, the skin was incubated in a water bath for 10 min at 37 °C while constant shaking. After incubation 4 °C cold PBS was added and the epidermis and dermis were separated using forceps. The epidermis was incubated in Trypsin-EDTA/PBS Solution (1:3) with constant shaking for 10 min at 37 °C. After adding 2 ml fetal calf serum (FCS) (PAN Biotech) the tissue mixture was filtered through a cell strainer (pore size: 100 µm), spun at 250 x g for 10 min and washed with PBS. Supernatant was discarded and the pellet was resuspended in keratinocyte culture medium (Keratinocyte-SFM, Gibco) supplemented by the corresponding manufacturer's supplements, 100 U/ml penicillin and 100 µg/ml streptomycin (PAN Biotech). Cells were

cultivated in 100 mm cell culture dishes under standard culture conditions (98% humidity, 37 °C, 5 % CO₂).

Medium was changed twice a week and cells were inspected daily with a light microscope. If cells reached approx. 70-80 % of confluence, they were sub-cultured, seeded or cryo-conserved. Cells were detached using a cell scraper after incubating in 2 ml 1 % trypsin/EDTA solution (Biowest) at 37 °C and neutralizing with 2 ml trypsin neutralizer (Gibco) after remaining 5 min in trypsin. After spinning at 300 x g for 5 min supernatant was discarded, cells were resuspended in keratinocyte culture medium and cell number was determined by using the Neubauer chamber.

For experiments cells (2×10^5 cells/well) were transferred to transparent six-well culture plates and were cultivated in 2 ml Keratinocyte Culture Medium/well for five days to achieve a confluent monolayer of cells. All measurements were performed with cells from passages one to four.

Irradiation of human keratinocytes with LED-arrays

According to specification of Philips Research the LED device was distance and power adjusted to deliver 38 W/cm². Keratinocytes were exposed to doses of 10, 20, 40 and 80 J/cm². During irradiation the phenol red-containing Keratinocyte Culture Medium in the six-well cell culture plates was replaced through colorless PBS. To avoid heat generation and vaporization of media during irradiation, the irradiated cell culture plates were cooled by ventilation and temperature of the media was measured to ensure to not exceed 35 °C. Control plates were treated similar to irradiated plates and remained outside the incubator at room temperature while irradiation. After irradiation PBS was replaced through keratinocyte culture medium and cells were incubated at 37 °C, 5% CO₂ until harvesting.

Quantification of S-nitroso-proteins in homogenates of human skin fibroblasts by CLD

Detection of intracellular concentrations of nitrite or nitroso compounds was performed with homogenates of human skin keratinocytes, which were prepared by detaching cells from culture dishes by trypsinization, diluting the pellet of 1×10^7 fibroblasts in 300 μ l NEM-buffer (PBS containing 5 mM N-ethyl-maleimide (NEM), 2.5 mM EDTA, protease inhibitor cocktail), and homogenizing the cell suspension by ultrasonification for 1 minute. After short centrifugation, supernatants were collected, diluted to a protein content of 10 mg/ml, and immediately used or frozen at -20°C for a maximum period of two weeks. Protein concentrations were determined using the Bio-Rad D_c Protein Assay Kits (Bio-Rad, Munich, Germany). All solutions were adjusted to pH 7.4. This pH level has been kept constant during the entire experiment.

Concentrations of S-nitroso species in homogenates of human skin were quantified by reductive denitroization of skin homogenate samples prepared in the presence or absence of N-ethyl-maleimide (NEM) using a mixture of iodine/iodide in glacial acetic acid and subsequent detection of the released NO by the CLD exactly as described [24]. Discrimination between S-nitrosated molecules (RS-NO) and other nitroso species was achieved by preincubation of sample aliquots with mercuric chloride (HgCl_2 ; 0.2%), which selectively cleaves S-NO bonds while preserving N-nitroso moieties, followed by sulfanilamide [22].

Quantitative RT-PCR

For quantitative RT-PCR cells were harvested 0 min, 30 min or 60 min post-irradiation. Medium was discarded and cells were washed with PBS, dislodged from the six-well plates using a cell scraper and transferred into micro-centrifuge tubes. After spinning at 10,000 rpm for 30 sec, supernatant was discarded and total RNA was purified from keratinocytes by

using RNeasy Plus Mini Kit (Quiagen) according to manufacturer's instructions. To remove contaminating DNA, DNase and divalent cations from RNA, Ambion DNA-free DNA Removal Kit was used. Absorbance measurements at 260 nm were performed on a spectrophotometer (Nano Drop ND-1000, Peqlap) to quantify RNA concentration. Total RNA was converted into cDNA using Omniscript-RT-Kit (Qiagen) and subsequently incubated in a thermal cycler at 37 °C for 60 minutes (MyCycler, BioRad).

For PCR reactions 10 ng/4µl cDNA of each sample were analyzed in triplicate and normalized to 18SrRNA using reactions mixtures containing SYBR green (12,5 µl) (Power SYBR Green, Applied Biosystems), DNase, RNase free water (for POMC-gene: 7 µl, for 18SrRNA: 8 µl) and upper and lower primers (each for POMC-gene: 0,75 µl, for 18srRNA: 0,25 µl). POMC-gene primer sequences were used as described elsewhere [25]. 18srRNA primer sequences were 5'-CATGGTGACCACGGGTGAC-3' as forward primer and 5'-TTCCTTGGATGTGGTAGCCG-3' as reverse primer.

Samples were heated at 95 °C for 10 min initially and amplified for 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C by 7300-Real Time PCR System (Applied Biosystems).

Western Blotting

For Western Blot analysis cells were harvested 30 min, 1 h, 2 h, 4 h and 8 h after blue light irradiation of the six-well plates. Adherent cells were washed with PBS, dislodged from tissue culture plate using a cell scraper and transferred into microcentrifuge tubes. After spinning at 10,000 rpm for 30 sec, supernatant was discarded and 30 µl of RIPA lysis buffer (Abcam: 50 mM Tris pH=8, 150 mM NaCl, 1% NP-40, 0.5 % Na-deoxycholat, 0.1% SDS) was added. By ultrasonic treatment cells were further lysed. The concentration of protein was measured using Micro BCA Protein Assay Kit (Thermo Scientific) as described in the instructions provided by the manufacturer and a spectrophotometer. 12% stacking gel and 3% separating gel were used. Samples of 30 µg protein were mixed with 20 % 2-

mercaptoethanol and 4 x Laemmli Sample Buffer (252 mM Tris-HCl, 40 % glycerol, 8 % SDS, 0.01 % bromophenol blue). After heating for 5 min at 100 °C, samples were loaded on SDS-PAGE and ran through the separating gel with low voltage of 60V and through the stacking gel with higher voltage of 130 V. For a further control a sample of murine brain tissue containing the pituitary gland was loaded on each gel. Using the Bio Rad's Trans-Blot Turbo device proteins were transferred within 25 min by 25 V and 2.5 A to a nitrocellulose membrane. All membranes were blocked with 5% bovine serum albumin (BSA) for one hour and incubated with the primary antibody in 5% BSA (1:1000) at 4 °C with gentle shaking, overnight. In order to detect POMC, PC 1/3, PC 2 and p53 we used Goat Polyclonal Anti-POMC Antibody (Novus Biologicals), Rabbit Polyclonal Anti-PC 1/3 Antibody (Cell Signaling), Rabbit Monoclonal Anti-PC 2 Antibody (Cell Signaling) and Mouse Monoclonal Anti-p53 Antibody (Thermo Scientific). After incubating, membranes were washed 3 x 5 min in TBS-T (0.1% Tween 20) and incubated with secondary antibody in 5 % TBS-T (1:1000) for one hour at room temperature. As secondary antibodies Goat Polyclonal Anti-Mouse Antibody, Rabbit Polyclonal Anti-Goat Antibody and Goat Polyclonal Anti-Rabbit Antibody (all Dako) were used. Membranes were washed again as described (3 x 5 min in TBS-T) and incubated in enhanced luminescence reagents (Thermo Fisher Scientific). To normalize protein loading, Mouse Monoclonal Anti-GAPDH Antibody (Novus Biologicals) was used incubating in 3 % BSA overnight at 4 °C.

β-endorphin-specific enzyme-linked immunosorbent assay (ELISA)

At time points indicated cell culture supernatants were collected and the β-endorphin content was quantified using a specific enzyme-linked immunosorbent assay kit (Cloud-Clone Corp, Katy, TX, USA), which was used in accordance to the manufacturer's instructions.

Fluorescence-spectroscopic and fluorescence-microscopic detection of NO in resting and irradiated (453 nm) keratinocytes *in-vitro*

Numerous more or less specific fluorescent dyes are available for research of the role of reactive oxygen and nitric oxide species in biological system, each of them bearing merits and weaknesses [26]. The cell-permeable reagent 3-Amino-4-(N-methylamino)-2',7'-difluorescein diacetate (DAF-FM DA, Alexis Corporation, Lausen, Switzerland) can be used for the quantitative detection of low levels of NO-adducts. It has been suggested that the primary reaction of DAF-2 does not occur directly with the nitric oxide radical but rather with its oxidation product N_2O_3 [27-29] that may be formed in solutions by autoxidation of NO in the presence of oxygen [27]. The compound is not fluorescent before reacting with the NO product to form a fluorescent benzotriazole. After entry into the cell, DAF-FM DA is converted by intracellular esterases into the water-soluble dye DAF-FM, which reacts with NO-adducts to form the fluorescing benzotriazole. Benzotriazole fluorescence can be measured by using an excitation wavelength of 488 nm and an emission wavelength of 505 nm to 530 nm.

Here we used DAF-FM for the indirect detection and quantification of blue light (453 nm)-induced NO generation in human skin keratinocytes *in-vitro*. The final DAF-FM DA concentration was 10 μ M (prepared from a 5 mM stock solution in DMSO) whereby DAF-FM DA was added to the keratinocyte cultures (1×10^5 keratinocytes per well of a six-well cell culture plate) prior light exposure and was present during irradiation (80 J/cm²). Immediately after irradiation culture supernatants were discarded, the cells were washed twice with 2 ml of PBS, 2 ml of growth medium was pipetted in each of the wells, and the fluorescent in the cultures was visualized in a fluorescence microscope. Alternatively, cells were lysed and 200 μ l of the lysate was transferred into a 96-well ELISA plate and the resulted fluorescence was quantified by a fluorescence photometer. Importantly, to grasp only the effect of non-enzymatically generated NO, keratinocyte cultures were incubated prior as well as during the

irradiation with blue light (453 nm, 80 J/cm²) with 1 mM of N⁵-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO), a non-selective inhibitor of all nitric oxide synthase (NOS) isoforms.

Detection of S-nitroso proteins by immunohistochemistry

In resting human skin keratinocyte cultures, fixed by glutaraldehyde (0.2% in TBS, pH 7.0) for 15 min at 4° in a moist chamber, followed by inhibition of endogenous peroxidase activity with 0.3% H₂O₂ in ethanol, S-nitrosation of proteins was examined using a S-nitrosocysteine-specific rabbit anti-serum. We routinely check the reliability of the antiserum on nitrosated albumin samples by Western blot. After blocking of unspecific binding with 0.5% BSA in TBS for 30 min and rinsing, specimens were incubated with the rabbit anti-S-nitrosocysteine anti-serum used in a 1:100 dilution in TBS (supplemented with 3% low-fat milk powder and 0.5% Tween 20, pH 7.4). As secondary antibody peroxidase-conjugated goat anti rabbit IgG was used in a final dilution of 1:30 for one hour in TBS. As a negative control, samples were incubated only with the second antibody, i.e. without the application of the first antibody. All steps were performed at 4°C. Nuclei were stained with hematoxylin for 1 min. Then, samples were incubated with 0.05% diaminobenzidine + 0.015% H₂O₂ for 5 minutes at room temperature. For light microscopy, cell samples were dehydrated, cleared with xylene and embedded in Eukitt as described previously [10, 30].

Impact of nitric oxide and hydrogen peroxide on β -endorphin synthesis of human skin keratinocytes *in-vitro*

In order to evaluate the role of nitric oxide (NO) and hydrogen peroxide (H₂O₂) on β -endorphin synthesis of human keratinocytes, human skin keratinocytes (2 x 10⁵ cells/well) were transferred to transparent six-well culture plates and were cultivated in 2 ml *Keratinocyte Culture Medium*/well. After reaching a confluent monolayer keratinocyte cultures were incubated for 1, 4 or 24 hours with the nitric oxide (NO)-releasing

Diethylenetriamine NONOate (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO) or the mother substance DETA (Diethylenetriamine) alone at concentrations indicated. Alternatively keratinocyte cultures were incubated for 1, 4 or 24 hours with hydrogen peroxide (H₂O₂) at concentrations indicated. At the mentioned time points of incubation cell culture supernatants were collected and the β -endorphin content of the supernatants was quantified by a specific ELISA.

Statistical Analysis

Data were analyzed and compared by Graph Pad Prism (GraphPad Prism 5.0 for Windows) using one-way ANOVA and two-way ANOVA with post-hoc Bonferroni multiple comparison test. The common significance level of 0.05 was used.

RESULTS

Blue light enhances β -endorphin production in human epidermal keratinocytes

Human skin keratinocyte cultures growing in confluence were irradiated once with blue light at 453 nm at doses of 20, 40 or 80 J/cm². As shown in Figure 1, supernatant β -endorphin concentrations were significantly elevated 4 h after irradiation with the dose of 20 J/cm² (n=11). β -endorphin levels also increased significantly 1, 3. And 4 hours after irradiation with 40 J/cm² (n=10-12). Six and eight hours after irradiation β -endorphin concentrations were lower as compared to values of supernatant samples that were collected three or four after irradiation with 40 J/cm² (Figure 1). No significant changes in β -endorphin concentrations were observed in keratinocytes cultures that were irradiated with 80 J/cm² (Figure 1).

Effects of blue light exposure on p53, POMC, PC1/2, and PC2 protein expression in human epidermal keratinocytes

It has been demonstrated previously that the tumor suppressor p53 is activated by UV-light and directly modulates the transcriptional activity of the POMC promoter in keratinocytes [5]. In order to determine whether blue light exposure might exert analogue effects, human keratinocyte cultures were irradiated with blue light (20, 40, or 80 J/cm²) and at time points indicated the protein expression of p53, POMC, PC1/2, and PC2 was detected using the Western blot technique.

Results shown in Figure 2 reveal that blue light exposition of keratinocytes led to an apparently albeit not significantly elevated p53 protein expression at the first four hours after irradiation (Figure 2A). Elevated p53 protein expression neither did correlate with POMC gene expression (data not shown) nor with POMC protein expression, as shown in Figure 2B. Furthermore, blue light irradiation had no significant effect on PC1/3 or PC2 protein expression, but 30 minutes after blue light exposure PC1/3 protein levels were up to two-fold

higher (Figure 2D) and PC2 protein expression levels were up to four-fold higher as compared to the non-irradiated control keratinocyte cultures (Figure 2C).

S-nitroso-protein content of human skin keratinocytes

The cytoplasm of resting human skin keratinocyte contains a significant amount of S-nitrosated proteins, which we could visualize by using a S-nitrosocysteine-specific rabbit anti-serum (Figure 3B). As quantified by CLD, human skin keratinocytes contain more than 50 pmol/mg protein of S-nitrosated proteins (Figure 3C). The intracellular value of this photolabile NO derivate could be significantly depleted by exposure to 100 J/cm² of 453 nm (Figure 3C).

Blue light (453 nm) induces intracellular nitric oxide and reactive oxygen species in human skin keratinocytes *in-vitro*

In order to evaluate the impact of blue light on intracellular NO production in human skin keratinocyte cultures were irradiated with a dose of 80 J/cm² of blue light at 453 nm. Intracellular NO formation, as consequence of blue light-induced decomposition of photolabile S-nitrosated proteins, was visualized and quantified by using the NO-adduct-specific fluorescent dye DAF-FM-DA

Fluorescence microscopic evaluations of the experiments reveal a strongly enhanced DAF-fluorescence signal in irradiated keratinocytes (Figure 3D) as compared to non-irradiated keratinocyte cultures (Figure 3F). After lysing the respective cell cultures with cold isopropanol we quantified the resulted fluorescence signal by a fluorescence spectrometer and normalized the values to the protein content of the respective sample. As shown in Figure 3F, the NO-specific fluorescence signal was significantly higher in blue light-irradiated keratinocyte cultures. Irradiation of the fluorescence dye by blue light did not have any influence on the fluorescence signals of the dye (data not shown).

Impact of exogenously applied nitric oxide and hydrogen peroxide on keratinocyte β -endorphin production

Taking into consideration that in exposed keratinocyte cultures blue light induced an significant enhancement of NO and EP, we here studied the impact of exogenously applied NO on β -endorphin formation of cultured keratinocytes (Figure 4A-C). As NO-Donor we used DETA/NO at concentrations indicated. Additionally, as blue light also is able to induce intracellular H_2O_2 production in irradiated cells we have evaluated the impact of H_2O_2 on β -endorphin secretion of primary human skin keratinocytes (Figure 4D-F).

We show in Figure 4 that incubation of keratinocyte cultures with the NO-Donor DETA/NO for one, four or 24 hours led to an apparent increase in β -endorphin secretion of the respective keratinocyte culture. At each of the three observation points β -endorphin secretion was significantly increased after incubation with 12,5 μ M DETA/NO. The mother substance DETA alone applied at the respective concentrations (data not shown) but also H_2O_2 at concentrations indicated (Figure 4D-F) did not exert any significant effects on β -endorphin secretion of the keratinocytes.

Blue light exposure initiates nitric oxide emanation from human skin *in-vivo*

In order to capture and quantify NO emanation from human skin during blue-light exposure, we used a stainless steel collection chamber with a light transparent front window for the accumulation of emanated gases from human skin.

As shown in Figure 5A, blue light led to a significant and nearly linear exposure-time-dependent emanation of NO from light-exposed human skin *in-vivo*. After switching off the device NO emanation from human skins dropped again to baseline values.

Effects of blue light on serum concentration of bioactive nitric oxide derivatives

Using a whole-body irradiation device, fourteen male healthy subjects were irradiated for 30 minutes with blue light (450 nm) with a dose of 72 J/cm², or as control they were sham treated by covering them during the irradiation with an opaque filter foil. Thirty minutes prior irradiation as well as at time points indicated after the light exposure blood samples were collected and serum concentrations of nitrosated compounds (RX-NO) which represent a bioactive transport form of nitric oxide.

As shown in Figure 5B, which contains measured values obtained in the context of a very recently published study describing the impact of blue light on blood pressure of healthy volunteers [11], whole body exposure to blue light for 30 minutes led to a significantly enhanced systemic concentration of nitrosated compounds in blood serum as compared to sham-treated subjects. Significant increases in systemic RX-NO concentration could be observed immediately after as well as up to 30 minutes after blue light exposure. Subsequently, the elevated RX-NO concentrations dropped back to baseline values.

***In-vivo* effects of blue light on serum β -endorphin concentrations and nitric oxide generation**

In the identical serum samples that we used for RX-NO detection as described above for Figure 5B, we quantified the systemic serum concentrations of β -endorphin using a specific ELISA. As shown in Figure 6, in serum samples obtained from blue light-irradiated subjects immediately after light exposure β -endorphin concentration was significantly higher as compared to serum samples collected prior blue light exposure but also as compared to serum samples obtained from respective sham treated test persons. In the following 120 minutes the serum β -endorphin concentrations dropped down but at time point 120 minutes after light exposure the β -endorphin serum concentration of blue light exposed subjects was still significantly higher than in the respective serum samples obtained from the sham treated control group.

DISCUSSION

In the present work we show with human skin keratinocyte cultures *in-vitro* that exposure of the cells to blue light with a maximum intensity at around 453 nm induced a significant increase in keratinocytic β -endorphin production. Interestingly, elevated β -endorphin formation in the light-exposed cell cultures was paralleled by significantly increased non-enzymatic generation of nitric oxide (NO), which points at a pivotal role of NO in the observed effect. Indeed, the sole exogenous application of NO to cultured keratinocytes significantly enhanced keratinocytic β -endorphin production as well. Moreover, *in-vivo*, whole body exposure of healthy subjects to visible light at $\lambda=453$ nm significantly enhanced both systemic serum concentration of bioactive NO-derivates as well as of β -endorphin. These data reveal that our observation of a systemic response of blue light exposure on elevated β -endorphin concentrations can be plausibly explained by two mechanisms or, most probably, a combination of them. First, in human skin or skin keratinocytes, respectively, blue light liberates nitric oxide from photo-labile NO metabolites. In human skin *in-vivo*, a relevant fraction of this NO leads to keratinocytic *de novo* synthesis of β -endorphin, which might induce local effects, or after its systemic distribution via the blood circulation might exert a systemic impact. An additional and probably parallel running mechanism comprises the systemic distribution of cutaneously generated NO in the form of fairly stable nitroso compounds. Hereinafter, these bioactive NO derivates might elicit additional β -endorphin synthesis in peripheral as well as central nervous system and pituitary gland.

The scientific basis of the proposed mechanisms is strongly supported by published data. Thus, in human skin enzyme-dependent production of NO potentially occurs in all cell types by at least one of the three members of the NO synthase family [31]. Some of the NO molecules formed remain at or close to the point of their origin in the form of more stable derivates, e.g. N- and S-nitroso compounds (RX-NO) or the oxidation products nitrite and nitrate. In healthy human skin nitrite and RX-NO can be found at 25- and 360-times higher

concentrations than in blood of healthy volunteers [24]. Due to the enormity of the “organ” skin, the human cutis represents the greatest storage for NO-derivates [24]. The last information insofar is of distinct relevance as *in-vivo* RX-NOs, especially S-nitroso compounds, exert NO-like activity [32]. More importantly, nitrite and RX-NOs represent photo-labile NO derivates, which upon exposure to electromagnetic radiation might decompose and release NO. Previously we could show that UVA₁-radiation (340 - 400 nm) led to an effective decomposition of cutaneous photolabile NO derivates, which *in-vivo* in human skin resulted in pronounced local NO-dependent biological responses, e.g. enhanced local blood flow [9]. Moreover, in the case of whole body UVA₁-irradiation this UVA-induced cutaneous NO generation led to a significantly enhanced systemic concentration of bioactive NO derivates and significantly increased systemic NO-dependent hemodynamic parameters, e.g. significant reduction of middle arterial blood pressure [9]. In analogy to reactions induced by UVA radiation, we recently screened the visible light spectrum for its potency to decompose photo-labile NO derivates. We found that in human skin blue light at a wavelength of approx. 450 nm also possesses a strong capacity for photo-decomposition of nitrite as well as RX-NO. Following identical molecular and physicochemical mechanisms as described for UVA, local irradiation of human skin with blue light was shown also to induce increases in non-enzymatic local NO formation that were accompanied by the typical NO-specific biological responses, e.g. erythema formation [10]. Furthermore, very recently whole-body exposure of healthy volunteers to blue light was shown to enhance systemic concentration of bioactive NO-derivates, and was accompanied by pronounced effects on systemic hemodynamics [11].

As already mentioned above, in several rat animal models *in-vivo* NO directly or indirectly was involved in the activation of the pituitary pro-opiomelanocortin system and thus might influence metabolic pathways leading to elevated secretory activity of β -endorphin [12, 13, 15-18]. All these findings support our postulated mechanism of action whereupon in humans

blue light-enhanced formation of bioactive NO derivatives might represent the causal mechanism of enhanced β -endorphin production in skin keratinocytes and/or in central as well as peripheral nervous system, respectively.

Due to the lack of data on the biological effect of blue light, our findings and the postulated mechanisms of the impact of blue light on keratinocytic as well as systemic β -endorphin generation only can be discussed in the context of present data obtained from studies covering the impact of ultra violet radiation (UVR) on β -endorphin metabolism. Unfortunately, especially in earlier publications concerning this research field, the information regarding the physical characteristics of the used UV sources was very mistaken. Often, the term UVR was used generically for ultraviolet radiation, without any further specification, whether authors used UVA or UVB radiation. However, this is essential because the mechanisms of action of the two types of energy-rich radiations are completely different. No matter, in all publications where authors verifiably used UVB, irradiation of cells with UVB *in-vitro* or exposure of animal or human subjects to UVB *in-vivo* led to a clear induction of *de-novo* synthesis of pro-opiomelanocortin (POMC)-derived peptides, including β -endorphin [7, 8, 25, 33-35]. The mechanism of UVB-induced β -endorphin synthesis seems to be very different from that of blue light: 1.) In contrast to blue light, UVB was shown to induce DNA damage in the skin and skin cells [36], 2.) in contrast to UVB, blue light neither did lead to significantly elevated gene nor protein expression levels of POMC, as described previously for UVB [5], 3.) in analogy to UVB, blue light exposure induced a clear but not significant increase in P53 protein expression that was maximal 30 and 60 minutes after the light exposure, 4.) in UVB-irradiated keratinocytes elevated β -endorphin concentrations were shown to appear 6 to 8 hours after irradiation [25, 34, 37] and in UVB-irradiated animals significantly enhanced β -endorphin levels could be detected 4 to 7 days after UVB exposure [7], whereas with blue light significantly enhanced β -endorphin levels were detectable as soon as 30 to 120 minutes after exposure. This time course was paralleled by increases in protein expression

of the pro-hormone convertases PC1/3 and PC2. Although blue light-elevated expression of PC1/3 and PC2 was not statistically significant, the resulting elevated pro-hormone convertase-activity had obviously been sufficient to effect the mentioned increases in β -endorphin formation.

The available information on the effect of UVA radiation on local or systemic β -endorphin production is not as clear as for UVB. On the one side UVA was shown to induce a rapid raise in keratinocytic β -endorphin production as well as significantly enhanced systemic β -endorphin levels in UVA-exposed humans *in-vivo* [6, 38, 39], but on the other side data obtained from other very accurately performed studies describe contrary findings after which UVA exposure of humans *in-vivo* had no detectable effects on serum β -endorphin concentrations [40, 41]. Here it is important to notice that the UVA doses used for both scenarios were nearly identical and the experimental setups were absolutely comparable.

Of course, the question arises about the therapeutic relevance of our findings. Are there any meaningful clinical applications for blue light? We are convinced that such applications exist.

Fell *et al.* demonstrated in their mouse model that β -endorphin easily crosses the blood-brain barrier and that skin-derived β -endorphin exerts systemic effects [7] including neurophysiological effects in central nervous system [2]. β -endorphin binds with high affinity to μ -opioid receptors in the nervous system and has an analgesic effect [2]. In addition, β -endorphin has an influence on immunological processes, wound healing processes, depressive illnesses [3, 42, 43], and increased performance in competitive sports [44, 45]. Furthermore, concerning the positive effects of β -endorphin on prevention of depressive disorders [46-48], it would be conceivable to apply blue light-emitting devices as supportive measure in the therapy of depressive disorder, for instant by improving the mood of patients. Furthermore, the irradiation of athletes with blue light could be successful in competitive sports, as a blue-light-induced increase in the level of β -endorphin might allow athletes to shift their voluntary performance limits. β -endorphin release in response to exercise has

been known and studied since at least the 1980s [49]. Studies have demonstrated that serum concentrations of β -endorphin increase in response to acute exercise and training [49]. Several publications have shown that enhanced serum β -endorphin levels, as result of excessive training, induced a raise of tolerance levels, lowered anxiety and pain thresholds, and led to the development of euphoria. [44, 45, 50].

Thus, the described blue light-induced effects that are mediated by bioactive NO-derivates bear a relevant clinical potential to serve as supportive measure in the therapy of several psychiatric disorders. Additionally, due to the positive influence of β -endorphin on the mental state and other psychological parameters of humans, blue light might exert great potential in the home or leisure and sports field. Of course, it has to be considered that some users may develop addictive behaviors as was shown for UVB radiation [7]. However, due to the physical properties of blue light, severe side effects and dermatological complications, as expected in the case when using UV radiation, are not expected.

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Conflict of Interest

There are no relationships with industry other than stated above. The authors state no conflict of interest.

Disclosures

The authors state no disclosures

Declaration

The experimental protocol and the use of human material have been approved by the local ethics committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf (study number: 3634). The study protocol of the clinical study was approved by the *Ethics Committee of the Heinrich Heine University Duesseldorf* and all volunteers gave written informed consent (Clinicaltrials.gov: NCT03226587). All experiments described here were conducted in compliance with the *Declaration of Helsinki Principles*.

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LEGENDS TO THE FIGURES

Figure 1

Blue light irradiation induces the release of β -endorphin in human dermal keratinocytes. Shown are values of relative β -endorphin concentrations (normalized to values obtained with the untreated control) of human skin keratinocyte cultures exposed to 20 (**white bars**), 40 (**gray bars**) or 80 J/cm² (**black bars**) of blue light with a maximum intensity at 453 nm. β -endorphin concentration was quantified by a specific ELISA. Bars represent mean $\pm$ SEM of 10-12 individual experiments.

**, $p < 0.5$*

FIGURE 2

Impact of blue light irradiation on p53, pro-opiomelanocortin (POMC), pro-hormone convertase 1/3 (PC1/3) as well as pro-hormone convertase 2 (PC2) protein expression of human dermal keratinocytes. Shown are values of relative protein concentration (related to the untreated control) of human skin keratinocyte cultures exposed to 20 (**white bars**), 40 (**gray bars**) or 80 J/cm² (**black bars**) of blue light with a maximum intensity at 453 nm. P53 (**A**), POMC (**B**), PC1/3 (**C**) as well as PC2 protein (**D**) levels were assessed by Western blot analysis and normalized to GAPDH expression. Bars represent the mean $\pm$ SEM of 10-12 individual experiments.

FIGURE 3

S-nitroso-protein content of resting human skin keratinocytes and non-enzymatic generation of nitric oxide in blue light (453 nm)-irradiated human skin keratinocytes *in vitro*. **A**, Control keratinocyte cultures (incubated with a secondary horse-radish peroxidase linked secondary antibody but not with the S-nitrosocysteine-specific rabbit anti-serum. **B**, S-nitrosated proteins (RS-NO) in resting human skin keratinocyte were detected by using a S-nitrosocysteine-specific rabbit anti-serum (brown signal). **C**, RS-NO-values (pmol/ mg protein) in genuine as well as blue light-irradiated (100 J/cm^2 of 453 nm) cultures of human skin keratinocytes. Bars represent mean $\pm$ SD of six individual experiments. *, $p < 0.05$ as compared to non-irradiated genuine keratinocyte cultures. **D - F**, In order to evaluate the impact of blue light on intracellular non-enzymatic NO production of human skin keratinocytes, keratinocyte cultures were irradiated with blue light (453 nm, 80 J/cm^2) in the presence of 1.0 mM L-NIO, an effective NO-Synthase inhibitor. Intracellular generation of NO was visualized (**D**, **E**) and quantified (**F**) by using the NO-specific fluorescent dye DAF-FM-DA. **D**, Fluorescence microscopic evaluation of non-irradiated keratinocyte culture (negative control). **E**, Fluorescence microscopic evaluation of blue light-irradiated (80 J/cm^2) keratinocyte culture. **F**, NO-specific DAF-fluorescence in lysates of non-irradiated (white bar) and blue light-irradiated (gray bar; 80 J/cm^2) keratinocyte cultures. The resulted fluorescence signal was normalized to the protein content of the respective sample. Bars represent the mean $\pm$ SD of six individual experiments. Irradiation of the fluorescence dye alone with blue light did not influence the fluorescence properties of the dye (data not shown).

*, $p < 0.05$ as compared to non-irradiated control

Figure 4

Impact of exogenously applied nitric oxide or hydrogen peroxide on keratinocytic β -endorphin production. **A - C**, Impact of nitric oxide at concentrations indicated, here applied in the form of the NO-Donor DETA/NO, on β -endorphin secretion of human skin keratinocyte cultures. Bars represent the mean $\pm$ SD of five individual experiments. The mother substance DETA alone applied at the respective concentrations (data not shown) did not exert any significant effects on β -endorphin secretion of the Keratinocytes. *, $p < 0.05$ as compared to non-treated controls (0 μ M DETA/NO). **D - F**, Impact of hydrogen peroxide (H_2O_2) at concentrations indicated on β -endorphin secretion in human skin keratinocyte cultures. Bars represent mean $\pm$ SD of three individual experiments.

Figure 5

Impact of blue light on nitric oxide emanation from human skin and serum concentration of bioactive nitric oxide derivatives *in vivo*. **A**, *Quantification of NO emanation from human skin during blue light exposure *in vivo*.* Irradiation (38 J/cm^2) was performed for time intervals indicated with blue light at 453 nm. In the sham treated group the transparent window of the chamber was covered with an opaque filter foil. Bars represent mean $\pm$ SD of seven individual experiments. Bars represent the amount of NO (ppb) that accumulated in a light transparent collection chamber during the indicated time intervals of blue light exposure (**3, 6, 9 and 12 min**). After 12 minutes the light device was switched off. In **A**: *, $p < 0.05$ as compared to non-irradiated skin (0 min); #, $p < 0.05$ as compared NO values reached after 12 min of irradiation. **B**, *Serum concentration of bioactive nitric oxide derivatives of blue light-irradiated subjects *in vivo*.* Thirteen male healthy subjects were whole-body irradiated for 30 minutes with blue light (72 J/cm^2 , 450 nm, gray bars). The control group (white bars) was sham treated by covering the subjects during the irradiation with an opaque filter foil. Thirty minutes prior irradiation (**-60 min**) as well as at time points indicated after light exposure blood samples were collected and serum concentration of nitrosated compounds (RX-NO) was quantified by chemoluminescence-detection (CLD). Results are presented as Tukey box plots with 25th and 75th percentile as limits of the boxes and a line indicating the median of 13 subjects. In **B**: *, $p < 0.05$ as compared to samples obtained from the respective sham treated group immediately after irradiation (**0 min**) or 30 min after irradiation, respectively.

Please notice that measured values used for Figure 5B have been published recently in the context of an other thematic issue [11].

Figure 6

Blue light exposure enhances serum β -endorphin concentrations *in vivo*. In the identical blood samples from thirteen male healthy subjects, who were whole-body irradiated for 30 minutes with 72 J/cm² of blue light at 450 nm that were used for RX-NO quantification as described for Figure 5B, serum concentrations of β -endorphin (pg/ml) were quantified using a β -endorphin-specific ELISA (gray bars). The control group (white bars) was sham treated by covering the subjects during the irradiation with an opaque filter foil. β -endorphin concentrations were detected in blood samples collected 30 minutes prior irradiation (**-60 min**) as well as at time points indicated after light exposure. Results are presented as Tukey box plots with 25th and 75th percentile as limits of the boxes and a line indicating the median of 13 subjects.

^{*}, $p < 0.05$ as compared samples obtained prior irradiation (**-60 min**) and samples obtained from the respective sham treated group immediately after irradiation (**0 min**).

[#], $p < 0.05$ as compared samples obtained prior irradiation (**-60 min**).

[&], $p < 0.05$ as compared to samples obtained from the respective sham treated group 2 hours after irradiation (**120 min**).

FIGURES

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Figure 6