Constitutive and UV-induced metabolism of melatonin in keratinocytes and cell-free systems

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ABSTRACT **Melatonin, which can be produced in the skin, exerts a protective effect against damage induced by UV radiation (UVR). We have investigated the effect of UVB, the most damaging component of UVR, on melatonin metabolism in HaCaT keratinocytes and in a cell-free system. Four metabolites were identified by HPLC and LC-MS: 6-hydroxymelatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), 2-hydroxymelatonin (the main intermediate between melatonin and AFMK), and 4-hydroxymelatonin. Concentrations of these photoproducts were directly proportional to UVR-dose and to melatonin substrate content, and their accumulation was time-dependent. The UVR-dependent increase of AFMK and 2-hydroxymelatonin was also detected in keratinocytes, where it was accompanied by simultaneous consumption of intracellular melatonin. Of note, melatonin and its two major metabolites, 2-hydroxymelatonin and AFMK, were also detected in untreated keratinocytes, neither irradiated nor preincubated with melatonin. Thus, intracellular melatonin metabolism is enhanced under exposure to UVR. The additional biological activity of these individual melatonin metabolites increases the spectrum of potential actions of the recently identified cutaneous melatoninergic system.—Fischer, T. W., Sweatman, T. W., Semak, L., Sayre, R. M., Wortsman, J., Slominski, A. Constitutive and UV-induced metabolism of melatonin in keratinocytes and cell-free systems. *FASEB J.* 20, E897–E907 (2006)**

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Melatonin is no longer considered exclusively a pineal hormone (1, 2), but a bioactive substance with extrapineal sites of synthesis (3–10). Thus, melatonin can act as a receptor-independent autocrine and paracrine antioxidant (11, 12), as a direct radical scavenger (11, 13), as an immunomodulator (14), and as an antiaging (15, 16) and anticarcinogenic factor (17) in physiological and pharmacological concentrations (18).

An important peripheral target of melatonin is the skin where it is organized in a melatoninergic system, fully expressed in humans (9, 10, 19, 20) and rodents (21–23). Melatonin biosynthesis in the skin requires uptake of the essential amino acid L-tryptophan and enzymatic formation of 5-hydroxytryptophan by tryptophan hydroxylase, which is dependent on (6R) 5,6,7,8-tetrahydrobiopterin (6-BH4) (20, 24–26). Decarboxylation and further multistep-synthesis requiring arylalkylamine-N-acetyltransferase (AANAT) and hydroxy-indol-O-methyltransferase (HIOMT) produces melatonin (10, 19). Functionally, both cultured human keratinocytes and rodent melanoma cells have been shown to phenotype properties sensitive to melatonin (27–30), suggesting that melatonin—endogenous or exogenous—could play a role in protection of the skin against environmental stressors such as UV radiation (UVR) (9). In fact, UVR is the most prominent causative factor in photoaging and skin cancer and is likely responsible for the increasing incidence of UV-related basal cell and squamous cell carcinoma (31, 32), as well as malignant melanoma (33) over the past decades. Being a strong protectant against UV-induced damage both in vitro (9, 10, 34, 35) and in vivo (36, 37), melatonin could ensure the survival of keratinocytes and their clonogenic capacity against UV-induced damage (38). Moreover, it has been shown in vivo that exogenous melatonin applied topically in a cream preparation can penetrate into the skin and build a depot in the upper layers of the epidermis (39) to supplement the protective effects of endogenous melatonin (9, 39).

However, controversy exists concerning the direct effect of UVR on melatonin; while some have postulated that UVR causes production of phototoxic melatonin products (40), others have identified protective effects of UV-induced melatonin degradants, classified as antioxidative or antiinflammatory (41–47). Of note, these studies were performed in cell-free systems or in

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cells not normally exposed to UVR such as neutrophils (43, 48) and macrophages (41). Obviously, the cell population of maximal interest concerning UVR exposure and its effects on melatonin are the skin keratinocytes, however, these have not been previously investigated. This is particularly important, because a melatoninergic system is fully expressed in the skin (9, 10).

In the present study we first performed assays of melatonin photoproducts using varying UV-doses in a cell-free system. Second, melatonin photoproducts were investigated in supernatants and cell lysates of keratinocytes incubated with melatonin and submitted to UVR exposure and third, the metabolism of endogenous melatonin in untreated keratinocytes (without melatonin preincubation) was analyzed, before and after exposure to UVR.

MATERIALS AND METHODS

Cell culture

HaCaT keratinocytes were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glucose, L-glutamine, pyridoxine hydrochloride (Life Technologies, Inc., Invitrogen Life Technologies, Carlsbad, CA), 10% FBS (Mediatech Inc., Herndon, VA), and 1% penicillin/streptomycin/amphotericin antibiotic solution (Sigma Chemical Co., St. Louis, MO). Cells were trypsinized from culture flasks and seeded in 10 cm Petri dishes (Corning Inc., Corning, NY) at a density of 10^6 cells/dish and incubated overnight. The next day, after confluence of 80–90% was reached, cells were washed once with PBS to remove remnants of media, and incubation with melatonin in PBS was performed for 30 min. Parallel control dishes were incubated with PBS without melatonin. After incubation, the Petri dishes were irradiated from below with UVR. For the investigation of melatonin uptake and metabolism, keratinocytes were incubated with melatonin for 30 min or 24 h; endogenous melatonin production in keratinocytes was investigated in the absence of previous melatonin incubation. For cell-free experiments, melatonin in pure PBS was irradiated with UVR.

Melatonin and HPLC standards

Melatonin was purchased from Sigma and dissolved in absolute ethanol and further diluted with PBS (final concentration of ethanol$<$0.2%). Melatonin solution was added to cells at concentrations of 10^{-3} and 10^{-6} M for a 30 min or 24 h incubation. Internal standards for HPLC were dissolved in absolute ethanol. These included N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), 6-hydroxymelatonin (6-OH-Mel), 5-methoxytryptamine (5-MT), 5-methoxy-3-indol acetic acid (5-MIAA), and 5-methoxytryptophol (5-MTphol). All reagents, except for AFMK, were purchased from Sigma. AFMK was produced as described previously (49). AFMK was then purified by HPLC and its identity confirmed by UV spectra (at λmax=231, 262, and 342 nm) and verified by the mass spectrometry (findings of molecular ion [M+H]+ at m/z 265 and fragment ions at m/z 237 ([(M–N-acetyl)+H]+) and m/z 178 ([(M–(N-acetyl+N-formyl))+H]+).

UV irradiation

Irradiation experiments were performed with a Bio-Rad UV transluminator 2000 (Bio-Rad Laboratories, Hercules, CA), calibrated as described previously (38). Briefly, a scanning double monochromator spectroradiometer (Model OL 754, Optronic Laboratories, Orlando, FL) was used to scan emission of wavelengths from 250 to 800 nm at 1 nm increments. The spectroradiometer had been calibrated with an NIST traceable tungsten-halogen spectral irradiance standard (Model 752–10E, Optronic) with a precision current source (Model 65, Optronic). An additional calibration module (Model 752–150, Optronic) measured both photometric gain and wavelength accuracy. Wavelength calibration and gain were established or verified before each experimental use. The UV source emission (**Fig. 1**) consisted primarily of UVB light (wavelength 280–320 nm; ~60%), with minor output in the UVA (320–400 nm) and UVC (120–280 nm) range (~30% and ~10%, respectively). Melatonin photoproducts generation was performed after irradiation at the UV doses of 25, 50, 75, or 100 mJ/cm^2, as indicated in the figures.

Collection and extraction of samples

Samples of melatonin prepared in a cell-free environment were incubated for 30 min prior to irradiation in parallel to melatonin-exposed keratinocytes and frozen at −80°C immediately after irradiation. Supernatants from HaCaT keratinocytes preincubated with melatonin and submitted to UVR were collected at 10, 40, 190, and 370 min after UVR exposure and frozen at −80°C pending analysis. Cell pellets contained in 100 μl PBS were collected by trypsinization and centrifugation and were also frozen (−80°C) until further processing.

Supernatants and cell-free melatonin-PBS samples (25 ml) were extracted twice with 3 volumes of methylene chloride, and the pooled sample dried in a vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland). Cell pellets were sonicated (Vibra Cell; Sonics and Materials, Danbury, CT) in 5 ml of ice-cold PBS and were subjected to organic extraction from 25 ml PBS, as outlined above. Sample processing was conducted entirely under conditions of low ambient light and extracts were frozen at −80°C pending HPLC analysis.

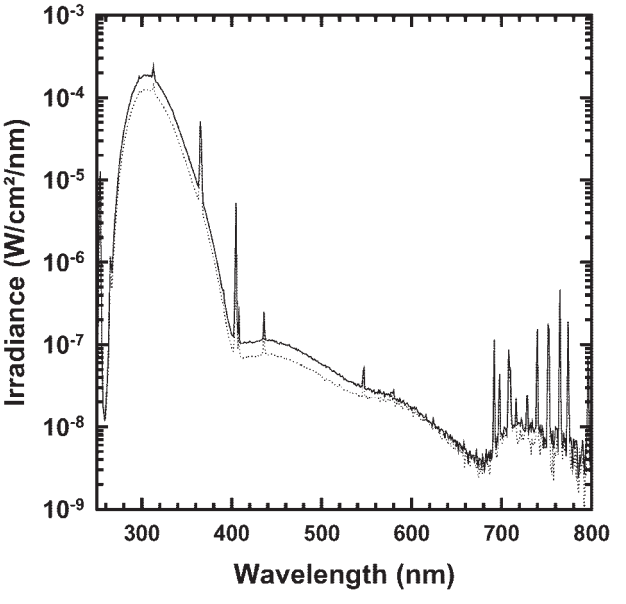


Figure 1. Emittance spectrum of the UV transluminator (Bio-Rad Model 2000) operating in normal (___) and preparative (. . . . .) mode. The maximum emittance is in the UVB range (280–320 nm).

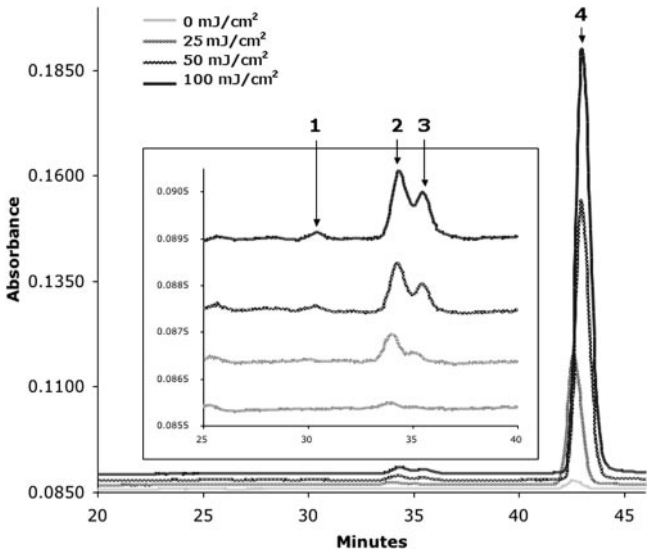


Figure 2. Detection of melatonin metabolites in a cell-free system measured by HPLC: 6-hydroxymelatonin (product 1), 2-hydroxymelatonin (product 2), 4-hydroxymelatonin (product 3), and AFMK (product 4). Product levels increased after irradiation of melatonin solution (10^{-3} M) in direct proportion to UV-doses (25, 50, 100 mJ/cm^2).

HPLC analysis

Cell extracts were briefly sonicated in 50 μl absolute ethanol in a Branson 5200 waterbath at ambient temperature (Branson Ultrasonics Corporation, Danbury, CT). The resuspended contents were transferred to 100 μl borosilicate glass vials (JP Cobert and Associates, St. Louis, MO) in a refrigerated (5°C) automated injector. HPLC analysis (all equipment, Waters Associates, Milford, MA) of 20 μl aliquots was accomplished with a C18 Nova-pak™ reverse-phase column (4 μm particle size; 10 cm × 5 mM id) using a gradient (5–15% over 40 min) of HPLC-grade acetonitrile (Fisher Scientific, Fairlawn, NJ) in phosphate buffer (0.01 M; pH 7.2) at 1.0 ml/min. Column eluate was monitored by Model 2487 UV detector (275 nm) and Model 991 photodiode array detectors, and the data were stored electronically for subsequent interpretation. Melatonin and related standards (in 5–20 μl absolute ethanol) were analyzed in an identical manner and identification of sample peaks made by correspondence to retention time, and where possible, absorption spectrum.

LC-MS analysis

Aliquots (20 ml) of samples from the cell suspension and cell-free experiments were separated on an LC–MS QP8000a (Shimadzu, Japan) equipped with diode array and single quadrupole mass-spectrometric detectors. The separation system consisted of a Restec Allure C18 reverse-phase column (150×4.6 mM; 5 μm particle size; and 60 A pore size) with mobile phase consisting of 25% acetonitrile and 0.1% acetic acid. Elution was performed isocratically at flow rate of 0.75 ml/min and temperature of 40°C. The eluent was routed to the mass-spectrometric electrospray interface (ESI) set in positive mode and using nitrogen as the nebulizing gas. Mass-spectrometry parameters were as follows: nebulizer gas flow rate, 4.5 l/min; electrospray voltage, 4.5 kV; and curved desolvation line (CDL) heater temperature, 250°C. The selected ion monitoring (SIM) mode was used to detect ions with m/z = 249 (monohydroxymelatonin); m/z = 265 (dihydroxymelatonin and AFMK); m/z = 237 (AMK); and m/z = 233 (melatonin). System control and data acquisition were performed with the LC–MS workstation Class-8000 software (Shimadzu, Japan).

RESULTS

Melatonin metabolites induced by UVR in a cell-free system

Irradiation of melatonin solution in a cell-free system (melatonin in PBS; UV-doses of 25, 50, and 100 mJ/cm^2) generated four compounds detected by HPLC at the retention times (RT) of 30 min (product 1), 34 min (product 2), 35 min (product 3), and 43 min (product 4). Peak areas increased linearly with UV-doses **(Fig. 2)**. Overall, product 4 showed the largest peak, followed by products 2, 3, and 1. In addition, peak size was also dependent on the preincubation concentrations of melatonin with higher concentrations of melatonin (10–3 M) producing larger photoproduct peaks as compared with the peaks from solutions containing melatonin at lower concentration (10–6 M). In the condition of lower preincubation concentration, only the major product 4 was detectable (data not shown). The UV dose-dependent increase of product 4 was further confirmed by LC-MS **(Fig. 3 *A-C*)**.

Product identity was determined by comparison with known standards of melatonin metabolites. Matches were found for product 1, 2, 3, and 4. Product 1 was identified as 6-hydroxymelatonin, product 2 as 2-hydroxymelatonin, product 3 as 4-hydroxymelatonin, and product 4 as AFMK **(Figs. 3–6)**.

Determinants of post-irradiation melatonin metabolism in keratinocyte supernatants

Supernatants of keratinocytes incubated with melatonin at the concentration of 10–3 M produced similar

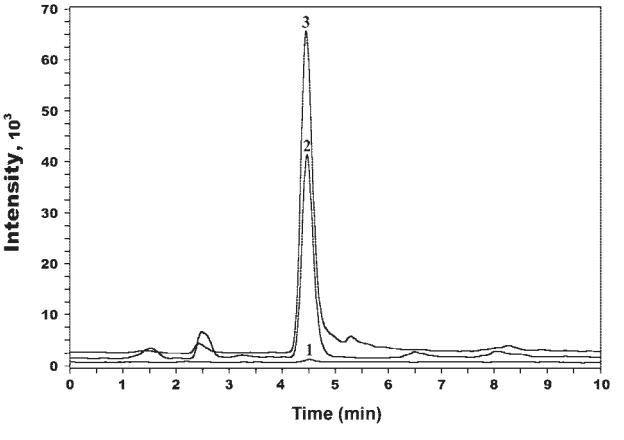


Figure 3. Baseline concentration (1) of AFMK (product 4) analyzed by LC-MS in extracts of melatonin solution (10^{-3} M), which has been irradiated with increasing UVR doses of 25 (2) and 75 mJ/cm^2 (3).

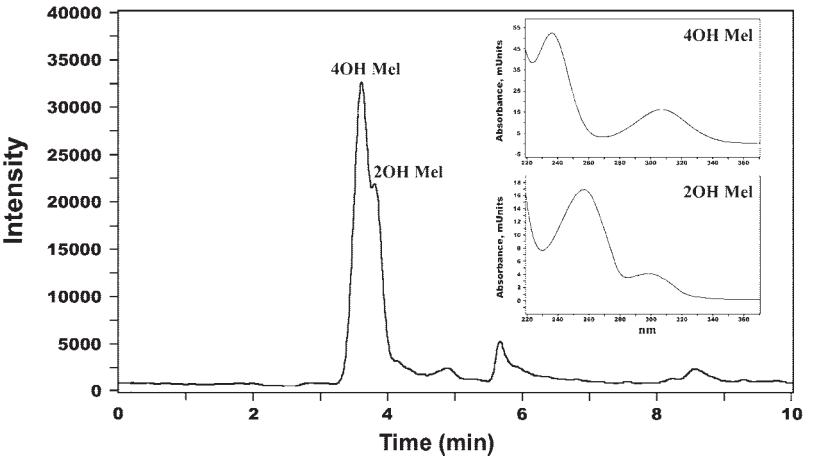


Figure 4. Detection of 2-hydroxymelatonin and 4-hydroxymelatonin by LC-MS (SIM mode for m/z=249) after irradiation of melatonin solution (cell-free system) with 75 mJ/cm^2. UV absorption spectra of the products (identical to the standards) confirm the structure of the compounds (see insets).

absorption spectra after irradiation with 25, 50, or 75 mJ/cm^2 with a UV-dose dependent effect. The metabolites detected in the supernatants corresponded to 2-hydroxymelatonin (product 2), 4-hydroxymelatonin (product 3), and AFMK (product 4) with the highest levels seen after irradiation with 75 mJ/cm^2. There was also a time effect, with larger increases in melatonin photoproducts in supernatants collected at later time points after UV exposure. Thus, metabolite levels were lowest immediately after UV irradiation and increased progressively thereafter. High levels of 2-hydroxymelatonin were detected as early as 40 min after UV exposure and remained elevated at 190 and 370 min post-UVR exposure **(Fig. 7*A*)**. Levels of 4-hydroxymelatonin were generally lower than those of 2-hydroxymelatonin, although 4-hydroxymelatonin increased steadily, similar to 2-hydroxymelatonin, from 40 to 370 min post-UV exposure (Fig. 7*B*). AFMK also

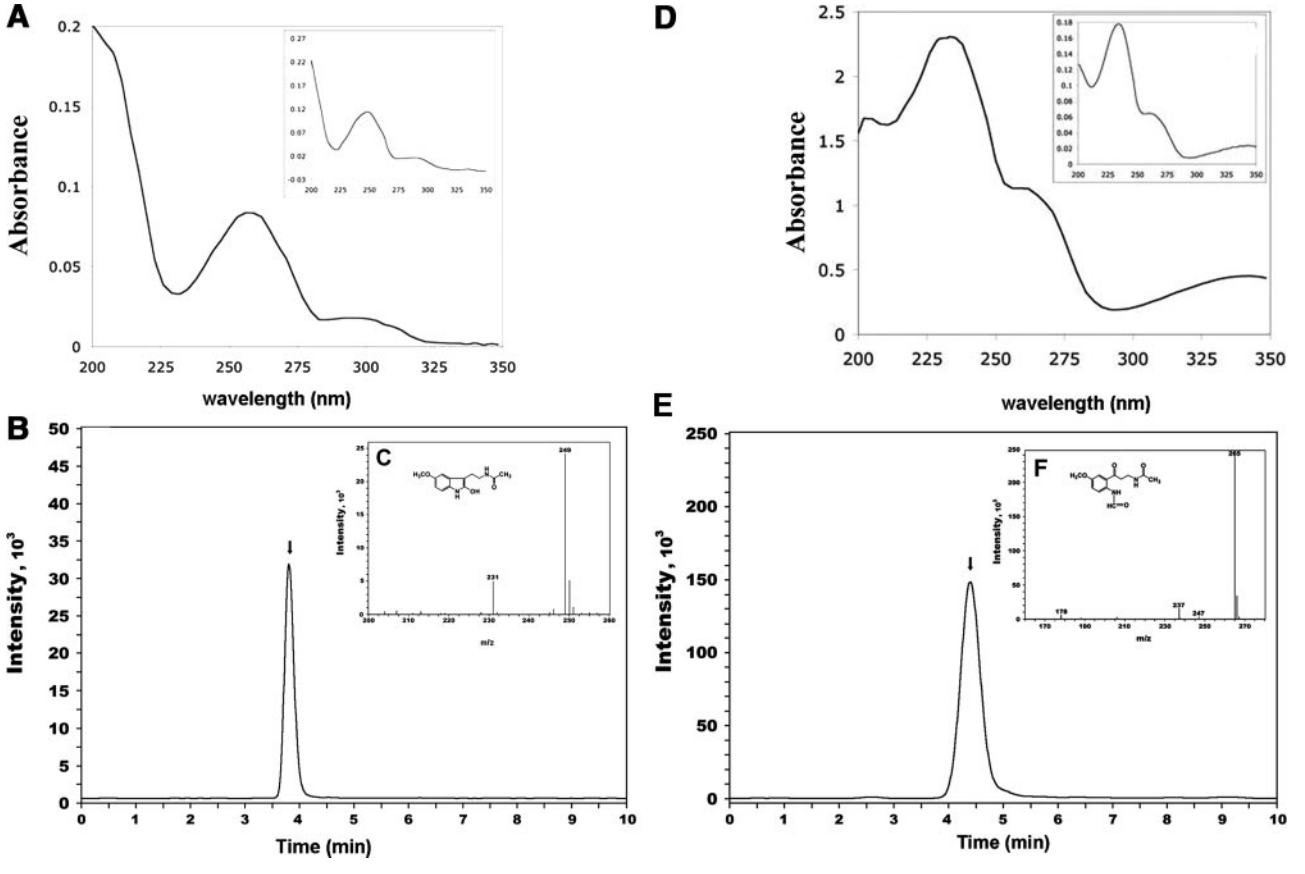


Figure 5. Identification of products 2 and 4. UV absorption curves of product 2 (RT 34 min) (*A*) and product 4 (RT 43 min) (*D*) are identical to absorption curves of corresponding standards of 2-hydroxymelatonin (inset in A) and AFMK (inset in *D*) analyzed by HPLC. LC-MS analysis in SIM mode (m/z=249 and m/z=265) further confirms that products 2 and 4 are 2-hydroxymelatonin (*B*; arrow) and AFMK (*E*; arrow), respectively. Mass spectra of the products and their chemical structure are presented in *C* and *F*, respectively.

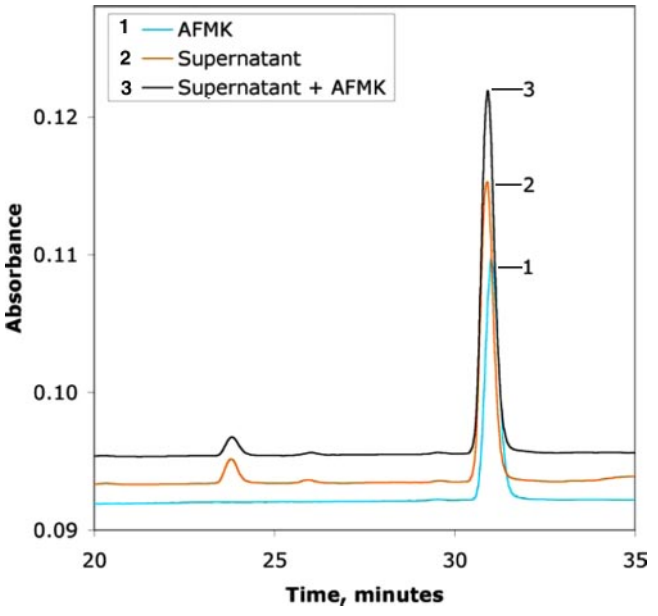


Figure 6. AFMK (product 4) detected as photoproduct after irradiation of melatonin solution at the dose of 25 mJ/cm^2, peaking at the same retention time as synthetic AFMK standard.

showed a time-dependent increase, with highest levels already reached at 40 min, to remain almost unchanged through 190 min and decreasing at 370 min (Fig. 7*C*). As in previous experiments with PBS (cell-free system), the overall AFMK levels were considerably higher than those of 2-hydroxymelatonin and 4-hydroxymelatonin.

Differential partition of melatonin metabolites

Whereas 2-hydroxymelatonin, 4-hydroxymelatonin and AFMK were identified in keratinocyte supernatants and cell lysates, 2-hydroxymelatonin was present at higher levels in the extracellular compartment, where it displayed strong increase after irradiation with 50 mJ/cm^2 (data not shown).

2-Hydroxymelatonin was nevertheless detectable intracellularly, but at very low levels, while still showing an increase after UVR exposure, as seen in the previous cell-free experiments; the post-UVR concentration was ~3-fold greater than the concentration in nonirradiated samples **(Fig. 8)**. 4-hydroxymelatonin was not detected intracellularly, neither under basal conditions (without UV irradiation) nor after UVR exposure. 4-Hydroxymelatonin was, however, detected in supernatants preincubated with melatonin and exposed to UVR (data not shown).

AFMK was detectable in supernatants as well as in cell lysates after melatonin preincubation, almost 100-fold higher in supernatants of irradiated samples (50 mJ/cm^2) compared with those nonirradiated **(Fig. 9*A*, *upper left inset*)**. Cell lysates generally showed lower levels of

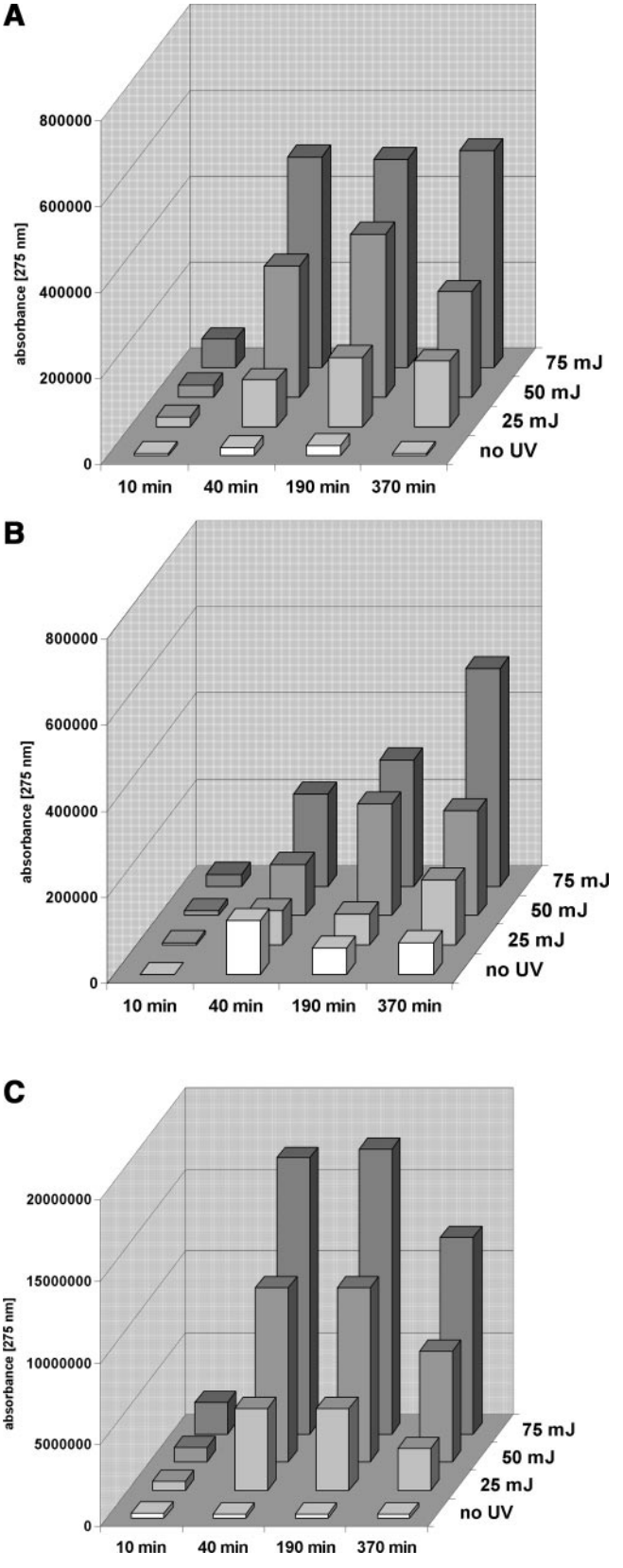


Figure 7. Kinetics of extracellular accumulations of 2-hydroxymelatonin (*A*), 4-hydroxymelatonin (*B*), and AFMK (*C*). HaCaT keratinocytes were preincubated with 10^{-3} M melatonin and exposed to UVR. Analysis of collected supernatants show that all products increased as early as 40 min after irradiation with 2- and 4-hydroxymelatonin remaining at high levels until 370 min post UVR, whereas AFMK showed a decrease after 190 min, indicative of further metabolism to AMK.

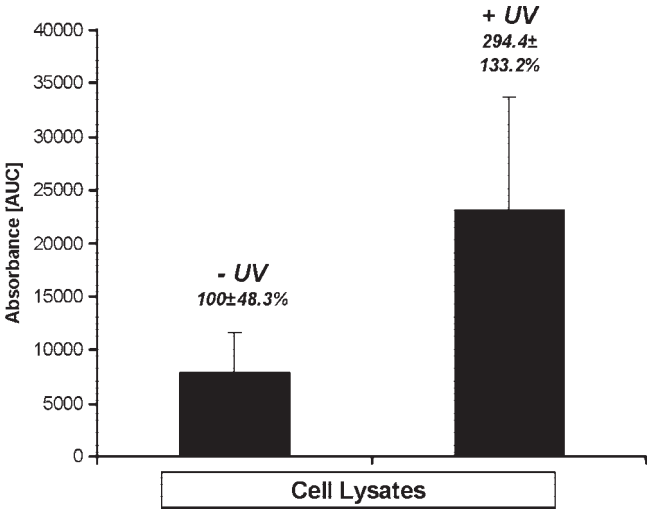


Figure 8. Relative levels of 2-hydroxymelatonin in HaCaT keratinocytes without melatonin preincubation. Irradiation with UVR (50 mJ/cm^2) leads to a 3-fold increase in intracellular 2-hydroxymelatonin concentrations compared to control. Columns represent means of absolute AUC-values of absorbance peaks from two experiments. Values above columns represent percentage mean ± SEM of unirradiated control.

AFMK than supernatants, but the UV-dependent increase was also observed. AFMK levels in lysates of cells irradiated with UVR were ~3.5-fold higher than the levels in nonirradiated lysates (Fig. 9*A*, *left*).

Interestingly, AFMK was still detected in supernatants of cells that had not been preincubated with melatonin, albeit at much lower levels. Moreover, the UV-dependent increase resulted in levels almost 13-fold higher compared with nonirradiated samples (Fig. 9*A*, *upper right inset*). AFMK was also detected in native keratinocytes not preincubated with melatonin, although at levels lower than in supernatants. Similar to the observations above, a distinct UV-stimulated increase of 1.9-fold was also evident (Fig. 9*A*, *right*).

Melatonin, the main substrate for AFMK production, was predictably detected at high levels in supernatants of samples preincubated with melatonin (10–3 M). These levels decreased slightly after UV exposure (to 99.7% of levels in the nonirradiated melatonin solution) (Fig. 9*B*, *inset*). Melatonin was not found in supernatants without melatonin preincubation. In contrast, cell lysates showed detectable levels of melatonin, even if the cells had not been incubated with melatonin (Fig. 9*B*, *right*). Cell lysates from keratinocytes preincubated with melatonin showed as expected higher melatonin levels than the lysates from cells not preincubated with melatonin (Fig. 9*B*, *left*). Nevertheless, with or without melatonin preincubation, cell lysates showed a decrease in melatonin levels after UV exposure (to 51.4 and 18.9% of unirradiated control, respectively) that was reciprocal to the increase of AFMK. Under basal conditions (without UV exposure), the ratio of intra- to extracellular melatonin of samples preincubated with melatonin was ~1:800 (0.125%).

Kinetics of melatonin metabolism in keratinocytes

Melatonin and its metabolites, AFMK and 2-hydroxymelatonin, were detected at low levels in cell lysates of untreated keratinocytes (not preincubated with melatonin). The intracellular concentration of melatonin in untreated keratinocytes was 146.0 pmoles/3×10^6 cells decreasing to 65.0 pmoles/3×10^6 cells after cultivation for 24 h (**Fig. 10*A***). Conversely, AFMK was detected at 17.4 pmoles/3×10^6 under basal conditions, increasing to 33.6 pmoles/3×10^6 cells after 24 h (Fig. 10*B*). Intracellular 2-hydroxymelatonin increased at 24 h, although to levels generally lower than those of AFMK and melatonin. The initial concentration of 2-hydroxymelatonin was 7.8 pmoles/3×10^6 cells; this increased to 20.4 pmoles/3×10^6 cells after 24 h (Fig. 10*C*). The metabolite 6-hydroxymelatonin was also detected (53.4 pmoles/3×10^6 cells) but decreased after 24 h (16.8 pmoles/3×10^6 cells; data not shown).

DISCUSSION

The present study describes melatonin metabolism in cell-free systems and cultured keratinocytes in vitro. Additionally, the effect of UVR exposure on this metabolism was studied. We found intense local metabolism with generation of 6-hydroxymelatonin, 2-hydroxymelatonin, 4-hydroxymelatonin, and AFMK. Furthermore, we could detect melatonin production in keratinocytes incubated in media free of melatonin. Thus, our studies uncover a novel functional significance for the cutaneous melatoninergic system.

In humans, 6-hydroxymelatonin is the chief metabolite of circulating melatonin, which is either endogenously produced by the pineal gland or of exogenous source by oral intake. Circulating melatonin is 6-hydroxylated through first-pass hepatic metabolism, further conjugated to 6-sulfatoxymelatonin and excreted in urine (50, 51). We did not find 6-hydroxymelatonin as a major product of UV-irradiation, although it was detected in non-UV-exposed keratinocytes and its intracellular levels decreased after 24 h of incubation. Thus, it is most likely that keratinocytes do metabolize melatonin to 6-hydroxymelatonin and may even have the capability to conjugate 6-hydroxymelatonin to 6-sulfatoxymelatonin, which is further released extracellularly. Indeed, Maharaj et al. (45) identified 6-hydroxymelatonin and AFMK after exposing melatonin in a cell-free system to UV–VIS (visible light wavelength) in a proportion of 1:2. In contrast, our study revealed 6-hydroxymelatonin to be only a minor product after UVR, which may be explained by the use of different UV wavelengths. However, we can confirm AFMK as the major product of melatonin degradation induced by selected UVR wavelengths. To date, AFMK has been detected only in the rat retina (52). In vitro, AFMK is generated by oxidation of melatonin, for example by reactive oxygen species (ROS) (53–55), which are produced at high levels after exposure to UVR. The

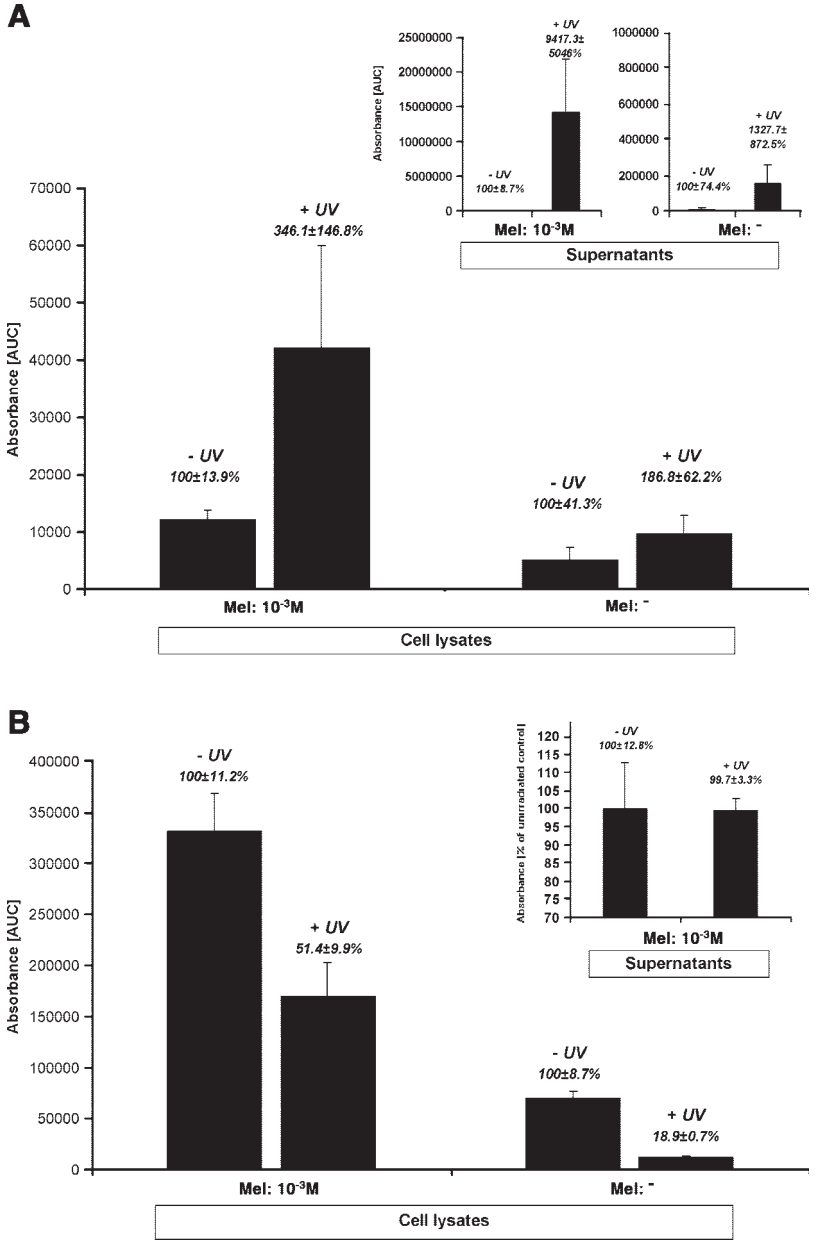
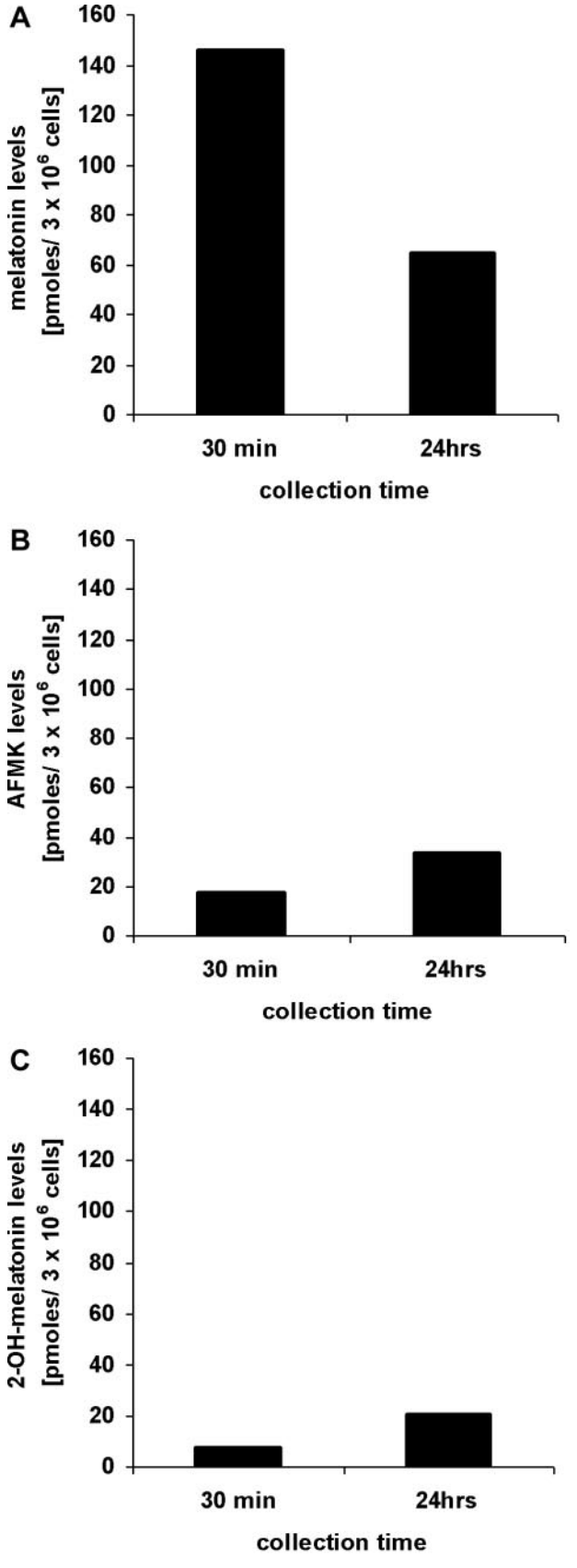


Figure 9. Detection of extra- and intracellular AFMK (*A*) and melatonin (*B*). The levels of AFMK were higher in keratinocytes preincubated with melatonin than in control cells (no melatonin added) (*A*). AFMK was also higher in supernatants that had been preincubated with melatonin, than without melatonin preincubation (*A*, *insets*). In both conditions and compartments, levels of AFMK increased after UV exposure. Melatonin, in contrast, showed reduced intracellular levels after UV exposure (*B*), whereas extracellular melatonin levels in the condition of melatonin preincubation showed no reduction (*B*, *inset*). Columns represent means of absolute AUC-values of absorbance peaks from two experiments. Values above columns represent percentage mean ± SEM of unirradiated control.

major and most damaging ROS, the hydroxyl radical, results from Fenton/Haber-Weiss reaction with hydrogen peroxide (56), and can be scavenged by melatonin, which is consecutively transformed to an indolyl cation radical and, in the presence of O_2^-, to AFMK (46). Also, the hydrogen peroxide itself can be directly scavenged by melatonin, which is then transformed into melatonin dioxetane and thereafter to AFMK (57). Both mechanisms appear to be relevant for AFMK formation in our study. Additionally, melatonin conversion to AFMK can involve oxidization by phorbol myristate acetate (PMA) and activation by lipopolysacharides (LPS) in leukocytes (48). Apart from those nonenzymatic mechanisms, AFMK production may be triggeed by cleavage of the melatonin pyrrole ring by indoleamine 2,3-dioxygenase (46), a major catabolic pathway of melatonin in tissues (58). Further metabolism of AFMK by arylamine formamidase leads to formation of N^1-acetyl-5-methoxykynuramine (AMK) (53). In leukocytes, melatonin is oxidized by myeloperoxidase and oxyferrylhemoglobin to AFMK (59, 60) that is further degraded to AMK by catalase (46). Recent studies have shown that AFMK can be also produced by oxidation of melatonin through mitochondrial cytochrome *c* (49).

The second major melatonin product detected post UVR exposure was 2-hydroxymelatonin previously identified only in Fenton-type OH-generating systems (61) or in reaction with hypochlorous acid (62). The cyclic form of 2-hydroxymelatonin has been detected in the jugular blood in the rat (63) and in human or rat urine after chloroform extraction (64) accounting for 5% of the urinary metabolites of melatonin. Since the levels of 6-hydroxymelatonin in the urine as well as in keratinocytes are much higher than those of 2-hydroxymelatonin, it can be concluded that 6-hydroxymelatonin could

be a major product when melatonin undergoes enzymatic metabolism, whereas 2-hydroxymelatonin would predominate during chemical reaction induced by UVR-related oxygen-based radicals (61) or by the combination of ROS with enzymes such as cytochrome *c* (49). In the present study we found 2-hydroxymelatonin to be a major intermediate between melatonin and AFMK following UVR exposure.



Product 3 was identified as 4-hydroxymelatonin. Interestingly, this product peaked shortly after 2-hydroxymelatonin when detected by HPLC, whereas the order of peak appearance in LC-MS was vice versa. This could be explained by use of different mobile phase for HPLC (neutral medium) and mass spectrometry (acid medium). Both products are generated after hydroxylation at carbon C2 and C4, and C2 seems to be the most favorable site for primary hydroxyl radical addition (65). The lower detection concentration of 2-hydroxymelatonin in LC-MS, however, may be explained by fast transformation of this product to 2,3-hydroxymelatonin and AFMK.

Previous studies on UVR-induced degradation of melatonin used light emissions in the UV–VIS range (wavelength 300–575 nm), with maximum emission in the UVA range at 365 and at 565 nm in the visible light range (45). In our study, the UV source emitted primarily in the UVB wavelength (280–320 nm), while a minor fraction in the UVA range (320–400 nm). Both wavelengths are important in cutaneous biology, since UVB causes the severe harmful effects in the epidermis, as represented by direct DNA damage in proliferating keratinocytes (66, 67) and generation of hydrogen peroxide by direct photochemistry, which leads to production of hydroxyl radicals by Fenton/Haber-Weiss reaction (56). Hydroxyl radicals react with melatonin at the carbon positions 2, 3, 4, and 6 (13) to build hydroxymelatonin molecules of which we could identify three (2-, 4-, and 6-hydroxymelatonin). UVA reaches deeper layers in the dermis causing fiber shrinkage and elastosis associated with skin aging (68). These processes are the major targets for the protective effects of melatonin, a strong radical scavenger, especially for the hydroxyl radical (15). Therefore, the same specific wavelengths were used to investigate the impact of UVR on melatonin itself. Of the resulting products, some have effects potentially protective: AFMK is known to be a strong radical scavenger, thus protecting against free radical formation, lipid peroxidation, and oxidative DNA damage (45, 46) and building an antioxidative cascade with melatonin and other melatonin metabolites (13). The increased formation of AFMK under progressively higher doses of UVR would therefore support the use of melatonin substrate in topically applied sun protective preparations, which can penetrate and build a depot in the upper layers of the skin (39). As a result, the organ could remain in equilibrium between the damaging effects of UVR and the protective effects of the UVR-

Figure 10. Relative levels of endogenous melatonin (*A*), AFMK (*B*), and 2-hydroxymelatonin (*C*) in HaCaT keratinocytes. Melatonin was detected in cells from control (no melatonin added) keratinocytes and its concentration decreased at 24 h, indicative of metabolic consumption (*A*). In contrast, intracellular levels of AFMK (*B*) and 2-hydroxymelatonin (*C*) increased after 24 h incubation.

induced increase of melatonin metabolites. This novel cutaneous defense mechanism may be defined as a melatoninergic antioxidative system (MAS).

Kinetic studies on the generation of photoproducts of melatonin in supernatants after UV exposure support their dual origin for cutaneous melatonin; thus, the progressive increase in 2-hydroxymelatonin and 4-hydroxymelatonin over the 6 h period may be explained not only by oxidation of melatonin in suspension but also by metabolization of intracellular melatonin stores with metabolite production and subsequent release into the extracellular compartment. Of further interest is the late decrease of AFMK at 3 to 6 h after UVR exposure that may be explained by additional metabolism of AFMK to AMK by arylamine formamidase. AMK, however, could not be detected, most likely due to very low levels. In a study by Silva et al. (48) the levels of AMK in activated leukocytes were 5 to 10% of AFMK since AMK, in contrast to AFMK, is easily oxidized.

To confirm the keratinocyte related production of 2-hydroxymelatonin and AFMK induced by UVR, we assayed for the products in cell lysates of keratinocytes and indeed found 2-hydroxymelatonin and AFMK. Also in analogy to the findings in the cell-free environment and supernatants, AFMK was the predominant metabolite. These observations indicate that the source of AFMK is intracellular melatonin. UVR-induced AFMK production was detected in both supernatants and cell lysates of keratinocytes not preincubated with melatonin; detectable intracellular melatonin levels were also evident. A melatonin source for AFMK production was further confirmed by the observation of still higher AFMK levels in supernatants and lysates of cells that had been preincubated with high concentration of melatonin (10–3 M). Since we could definitely detect melatonin in cell lysates, we could demonstrate that melatonin is consumed following UV irradiation. This observation was consistent in supernatants and cell lysates. Also, as expected, the intracellular melatonin levels were higher after preincubation with exogenous melatonin.

Even though melatonin is highly lipophilic and assumed to penetrate easily through lipid membranes (69), the uptake of melatonin into the cell was only 0.125% of applied levels. This low percentage is in agreement with data of Nickel and Wohlrab (70), who found melatonin uptake within the same range (0.097%) in HaCaT keratinocytes. The slight difference found between the two studies might be due to measurement of only the added tracer ([^{3}H]-labeled melatonin) by Nickel and Wohlrab, while our measurements included the endogenous pool plus the melatonin taken up from supernatants. Additional factors influencing melatonin uptake by HaCaT keratinocytes may be differences in culture conditions. In our experiments, keratinocytes were cultured in media containing 10% FBS, which may increase cellular metabolic activity and uptake of melatonin as compared to keratinocytes cultured in media with lower serum content or serum of different origin. For HaCaT keratinocytes grown under the described conditions, we can therefore safely conclude that addition of melatonin at 10–3 M will lead to intracellular melatonin levels at the concentration of ~10–6 M. These intracellular levels detected in HaCaT keratinocytes are still considerably higher than the levels of melatonin in human plasma, which gives another example confirming that melatonin is not in an equilibrium within an organism and that it can show considerable differences in its levels dependent on the compartment in which it is measured. In liquid compartments such as the bile, the bone marrow or cerebrospinal fluid (CSF), melatonin concentrations have been shown to be by orders-of-magnitude higher than in the plasma (5, 71–73). Also in cells, endogenous melatonin levels can differ significantly from the plasma when the cell populations have production rates that meet their requirements, e.g., in the gastrointestinal tract (3, 74), blood cells (6, 75) and retina (18, 76). Physiological vs. pharmacological levels of melatonin had been recently discussed (18), and it has been suggested that the physiological concentration of melatonin has to be defined at the local concentration, dependent on the specific cell, fluid, or organelle type (77). In this regard, we provide the first measurements of intracellular melatonin levels in HaCaT keratinocytes. Both, the presence of a functionally active melatoninergic system in the skin (9, 10, 19, 27,

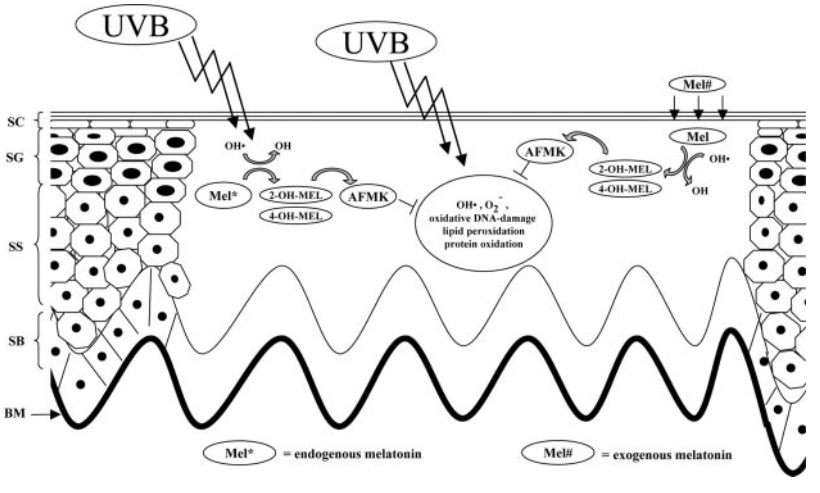


Figure 11. The melatoninergic antioxidative system (MAS) of the skin. Parallel to scavenging UVB-induced ROS, namely hydroxyl radicals, melatonin is also transformed to 2-hydroxymelatonin, 4-hydroxymelatonin, and consecutively to AFMK. AFMK is a potent free radical scavenger and, therefore, by itself can protect the skin against lipid peroxidation, protein oxidation, and oxidative DNA damage. The endogenous melatoninergic system can be supported by exogenous application of melatonin, which penetrates easily through the stratum corneum into deeper layers of the skin. SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; BM, basement membrane.

78) and the detection of melatonin production in human and murine hair follicles (79) support our present findings. We also have shown recently, that melatonin at the same concentration as used in the present study (10–3 M) protects cultured HaCaT keratinocytes against UVR-induced damage (38).

To conclude, we have shown that, within 24 h, keratinocytes metabolize melatonin to AFMK and 6-hydroxymelatonin as major products, with concomitant increase of 2-hydroxymelatonin, the intermediate between melatonin and AFMK, while melatonin consumption is detected. Most importantly, this process can be directly activated by UVR. Thus, melatonin may play an important role in cutaneous biology by protecting the skin against solar radiation. Moreover, generation of the UV-induced melatonin metabolites, which are strong antioxidants and therefore protective substances themselves, defines a novel melatoninergic antioxidative system (MAS) of the skin **(Fig. 11)**. Finally, the combination of endogenous melatonin with externally applied melatonin may successfully counteract the multiple processes of skin damage induced by UVR. Fj

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REFERENCES

1. Arendt, J. (1988) *Melatonin. Clin. Endocrinol. (Oxf)* **29,** 205–229
2. Lerner, A. B., Case, J. D., Takahashi, Y. (1958) Isolation of melatonin, a pineal factor that lightens melanocytes. *J. Am. Chem. Soc.* **80,** 2587
3. Bubenik, G. A. (2002) Gastrointestinal melatonin: localization, function, and clinical relevance. *Dig. Dis. Sci.* **47,** 2336–2348
4. Cahill, G. M., and Besharse, J. C. (1992) Light-sensitive melatonin synthesis by Xenopus photoreceptors after destruction of the inner retina. *Vis. Neurosci.* **8,** 487–490
5. Tan, D. X., Manchester, L. C., Reiter, R. J., Qi, W. B., Zhang, M., Weintraub, S. T., Cabrera, J., Sainz, R. M., and Mayo, J. C. (1999) Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. *Biochim. Biophys. Acta* **1472,** 206–214
6. Carrillo-Vico, A., Calvo, J. R., Abreu, P., Lardone, P. J., Garcia-Maurino, S., Reiter, R. J., and Guerrero, J. M. (2004) Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. *FASEB J.* **18,** 537–539
7. Itoh, M. T., Ishizuka, B., Kuribayashi, Y., Amemiya, A., and Sumi, Y. (1999) Melatonin, its precursors, and synthesizing enzyme activities in the human ovary. *Mol. Hum. Reprod.* **5,** 402–408
8. Reiter, R. J., and Tan, D. X. (2002) Melatonin: an antioxidant in edible plants. *Ann. N. Y. Acad. Sci.* **957,** 341–344
9. Slominski, A., Fischer, T. W., Zmijewski, M. A., Wortsman, J., Semak, I., Zbytek, B., Slominski, R. M., and Tobin, D. J. (2005) On the role of melatonin in skin physiology and pathology. *Endocrine* **27,** 137–148
10. Slominski, A., Wortsman, J., and Tobin, D. J. (2005) The cutaneous serotoninergic/melatoninergic system: securing a place under the sun. *FASEB J.* **19,** 176–194
11. Tan, D. X., C. L., Poeggeler, B., Manchester, L. C., Reiter, R. J. (1993) Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr. J.* **1,** 57–60
12. Tan, D. X., Manchester, L. C., Hardeland, R., Lopez-Burillo, S., Mayo, J. C., Sainz, R. M., and Reiter, R. J. (2003) Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *J. Pineal. Res.* **34,** 75–78
13. Tan, D. X., Reiter, R. J., Manchester, L. C., Yan, M. T., El Sawi, M., Sainz, R. M., Mayo, J. C., Kohen, R., Allegra, M., and Hardeland, R. (2002) Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr. Top. Med. Chem.* **2,** 181–197
14. Maestroni, G. J. (2001) The immunotherapeutic potential of melatonin. *Expert. Opin. Investig. Drugs* **10,** 467–476
15. Reiter, R. J., Tan, D. X., Poeggeler, B., Menendez-Pelaez, A., Chen, L. D., and Saarela, S. (1994) Melatonin as a free radical scavenger: implications for aging and age-related diseases. *Ann. N. Y. Acad. Sci.* **719,** 1–12
16. Karasek, M., and Reiter, R. J. (2002) Melatonin and aging. *Neuroendocrinol. Lett. 23 Suppl.* **1,** 14–16
17. Karbownik, M. (2002) Potential anticarcinogenic action of melatonin and other antioxidants mediated by antioxidative mechanisms. *Neuroendocrinol. Lett. 23 Suppl.* **1,** 39–44
18. Reiter, R. J., Tan, D. X., and Maldonado, M. D. (2005) Melatonin as an antioxidant: physiology versus pharmacology. *J. Pineal. Res.* **39,** 215–216
19. Slominski, A., Pisarchik, A., Semak, I., Sweatman, T., Wortsman, J., Szczesniewski, A., Slugocki, G., McNulty, J., Kauser, S., Tobin, D. J., Jing, C., and Johansson, O. (2002) Serotoninergic and melatoninergic systems are fully expressed in human skin. *FASEB J.* **16,** 896–898
20. Slominski, A., Semak, I., Pisarchik, A., Sweatman, T., Szczesniewski, A., and Wortsman, J. (2002) Conversion of L-tryptophan to serotonin and melatonin in human melanoma cells. *FEBS Lett.* **511,** 102–106
21. Slominski, A., Pisarchik, A., Semak, I., Sweatman, T., and Wortsman, J. (2003) Characterization of the serotoninergic system in the C57BL/6 mouse skin. *Eur. J. Biochem.* **270,** 3335–3344
22. Slominski, A., Pisarchik, A., and Wortsman, J. (2004) Expression of genes coding melatonin and serotonin receptors in rodent skin. *Biochim. Biophys. Acta* **1680,** 67–70
23. Slominski, A., Baker, J., Rosano, T. G., Guisti, L. W., Ermak, G., Grande, M., and Gaudet, S. J. (1996) Metabolism of serotonin to N-acetylserotonin, melatonin, and 5-methoxytryptamine in hamster skin culture. *J. Biol. Chem.* **271,** 12281–12286
24. Schallreuter, K. U., Wood, J. M., Pittelkow, M. R., Gutlich, M., Lemke, K. R., Rodl, W., Swanson, N. N., Hitzemann, K., and Ziegler, I. (1994) Regulation of melanin biosynthesis in the human epidermis by tetrahydrobiopterin. *Science* **263,** 1444–1446
25. Hasse, S., Gibbons, N. C., Rokos, H., Marles, L. K., and Schallreuter, K. U. (2004) Perturbed 6-tetrahydrobiopterin recycling via decreased dihydropteridine reductase in vitiligo: more evidence for H2O2 stress. *J. Invest. Dermatol.* **122,** 307–313
26. Slominski, A., Pisarchik, A., Johansson, O., Jing, C., Semak, I., Slugocki, G., and Wortsman, J. (2003) Tryptophan hydroxylase expression in human skin cells. *Biochim. Biophys. Acta* **1639,** 80–86
27. Slominski, A., Pisarchik, A., Zbytek, B., Tobin, D. J., Kauser, S., and Wortsman, J. (2003) Functional activity of serotoninergic and melatoninergic systems expressed in the skin. *J. Cell. Physiol.* **196,** 144–153
28. Hipler, U. C., Fischer, T. W., and Elsner, P. (2003) HaCaT cell proliferation influenced by melatonin. *Skin Pharmacol. Appl. Skin Physiol.* **16,** 379–385
29. Slominski, A., and Pruski, D. (1993) Melatonin inhibits proliferation and melanogenesis in rodent melanoma cells. *Exp. Cell Res.* **206,** 189–194
30. Kadekaro, A. L., Andrade, L. N. S., Floeter-Winter, L. M., Rollag, M. D., Virador, V., Vieira, W., and Castrucci, A. M. (2004) MT-1 melatonin receptor expression increases the antiproliferative effect of melatonin on S-91 murine melanoma cells. *J. Pineal. Res.* **36,** 204–211
31. Berman, B. (2001) Basal cell carcinoma and actinic keratoses: patients' perceptions of their disease and current treatments. *Int. J. Dermatol.* **40,** 573–576
32. Collins, G. L., Nickoonahand, N., and Morgan, M. B. (2004) Changing demographics and pathology of nonmelanoma skin cancer in the last 30 years. *Semin. Cutan. Med. Surg.* **23,** 80–83
33. de Vries, E., Bray, F. I., Eggermont, A. M., Coebergh, J. W., and Registries., E. N. O. C. (2004) Monitoring stage-specific trends in melanoma incidence across Europe reveals the need for more complete information on diagnostic characteristics. *Eur. J. Cancer Prev.* **13,** 387–395

34. Fischer, T. W., Scholz, G., Knoll, B., Hipler, U. C., and Elsner, P. (2001) Melatonin reduces UV-induced reactive oxygen species in a dose-dependent manner in IL-3-stimulated leukocytes. *J. Pineal Res.* **31,** 39–45
35. Fischer, T. W., Scholz, G., Knoll, B., Hipler, U. C., and Elsner, P. (2004) Melatonin suppresses reactive oxygen species induced by UV irradiation in leukocytes. *J. Pineal. Res.* **37,** 107–112
36. Bangha, E., Elsner, P., and Kistler, G. S. (1997) Suppression of UV-induced erythema by topical treatment with melatonin (N-acetyl-5-methoxytryptamine). Influence of the application time point. *Dermatology* **195,** 248–252
37. Dreher, F., Gabard, B., Schwindt, D. A., and Maibach, H. I. (1998) Topical melatonin in combination with vitamins E and C protects skin from ultraviolet-induced erythema: a human study in vivo. *Br. J. Dermatol.* **139,** 332–339
38. Fischer, T. W., Zbytek, B., Sayre, R. M., Apostolov, E. O., Basnakian, A. G., Sweatman, T. W., Wortsman, J., Elsner, P., and Slominski, A. (2006) Melatonin increases survival of HaCaT keratinocytes by suppressing UV-induced apoptosis. *J. Pineal. Res.* **40,** 18–26
39. Fischer, T. W., Greif, C., Fluhr, J. W., Wigger-Alberti, W., and Elsner, P. (2004) Percutaneous penetration of topically applied melatonin in a cream and an alcoholic solution. *Skin Pharmacol. Physiol.* **17,** 190–194
40. Kim, Y. O., Chung, H. J., Chung, S. T., Kim, J. H., Park, J. H., Kil, K. S., and Cho, D. H. (1999) Phototoxicity of melatonin. *Arch. Pharm. Res.* **22,** 143–150
41. Mayo, J. C., Sainz, R. M., Tan, D. X., Hardeland, R., Leon, J., Rodriguez, C., and Reiter, R. J. (2005) Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. *J. Neuroimmunol.* **165,** 139–149
42. Onuki, J., Almeida, E. A., Medeiros, M. H., and Di Mascio, P. (2005) Inhibition of 5-aminolevulinic acid-induced DNA damage by melatonin, N1-acetyl-N2-formyl-5-methoxykynuramine, quercetin or resveratrol. *J. Pineal Res.* **38,** 107–115
43. Silva, S. O., Rodrigues, M. R., Ximenes, V. F., Bueno-da-Silva, A. E., Amarante-Mendes, G. P., and Campa, A. (2004) Neutrophils as a specific target for melatonin and kynuramines: effects on cytokine release. *J. Neuroimmunol.* **156,** 146–152
44. Ressmeyer, A. R., Mayo, J. C., Zelosko, V., Sainz, R. M., Tan, D. X., Poeggeler, B., Antolin, I., Zsizsik, B. K., Reiter, R. J., and Hardeland, R. (2003) Antioxidant properties of the melatonin metabolite N1-acetyl-5-methoxykynuramine (AMK): scavenging of free radicals and prevention of protein destruction. *Redox Rep.* **8,** 205–213
45. Maharaj, D. S., Anoopkumar-Dukie, S., Glass, B. D., Antunes, E. M., Lack, B., Walker, R. B., and Daya, S. (2002) The identification of the UV degradants of melatonin and their ability to scavenge free radicals. *J. Pineal. Res.* **32,** 257–261
46. Tan, D. X., Manchester, L. C., Burkhardt, S., Sainz, R. M., Mayo, J. C., Kohen, R., Shohami, E., Huo, Y. S., Hardeland, R., and Reiter, R. J. (2001) N1-acetyl-N2-formyl-5-methoxykynuramine, a biogenic amine and melatonin metabolite, functions as a potent antioxidant. *FASEB J.* **15,** 2294–2296
47. Kelly, R. W., Amato, F., and Seamark, R. F. (1984) N-acetyl-5-methoxy kynurenamine, a brain metabolite of melatonin, is a potent inhibitor of prostaglandin biosynthesis. *Biochem. Biophys. Res. Commun.* **121,** 372–379
48. Silva, S. O., Rodrigues, M. R., Carvalho, S. R., Catalani, L. H., Campa, A., and Ximenes, V. F. (2004) Oxidation of melatonin and its catabolites, N1-acetyl-N2 -formyl-5-methoxykynuramine and N1-acetyl-5-methoxykynuramine, by activated leukocytes. *J. Pineal Res.* **37,** 171–175
49. Semak, I., Naumova, M., Korik, E., Terekhovich, V., Wortsman, J., and Slominski, A. (2005) A novel metabolic pathway of melatonin: oxidation by cytochrome *c*. *Biochemistry* **44,** 9300–9307
50. Ma, X., Idle, J. R., Krausz, K. W., and Gonzalez, F. J. (2005) Metabolism of melatonin by human cytochromes p450. *Drug Metab. Dispos.* **33,** 489–494
51. Lerner, A. B., and Nordlund, J. J. (1978) Melatonin: clinical pharmacology. *J. Neural. Transm. Suppl.*, **13,** 339–347
52. Rozov, S. V., Filatova, E. V., Orlov, A. A., Volkova, A. V., Zhloba, A. R., Blashko, E. L., and Pozdeyev, N. V. (2003) N1-acetyl-N2-formyl-5-methoxykynuramine is a product of melatonin oxidation in rats. *J. Pineal Res.* **35,** 245–250
53. Hardeland, R., Reiter, R. J., Poeggeler, B., and Tan, D. X. (1993) The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. *Neurosci. Biobehav. Rev.* **17,** 347–357
54. de Almeida, E. A., Martinez, G. R., Klitzke, C. F., de Medeiros, M. H., and Di Mascio, P. (2003) Oxidation of melatonin by singlet molecular oxygen (O2(1deltag)) produces N1-acetyl-N2-formyl-5-methoxykynurenine. *J. Pineal Res.* **35,** 131–137
55. Almeida, E. A., Klitzke, C. F., Martinez, G. R., Medeiros, M. H., and Di Mascio, P. (2004) Synthesis of internal labeled standards of melatonin and its metabolite N1-acetyl-N2-formyl-5-methoxykynuramine for their quantification using an on-line liquid chromatography-electrospray tandem mass spectrometry system. *J. Pineal Res.* **36,** 64–71
56. Schallreuter, K. U., and Wood, J. M. (2001) Thioredoxin reductase–its role in epidermal redox status. *J. Photochem. Photobiol. B* **64,** 179–184
57. Tan, D. X., Manchester, L. C., Reiter, R. J., Plummer, B. F., Limson, J., Weintraub, S. T., and Qi, W. (2000) Melatonin directly scavenges hydrogen peroxide: a potentially new metabolic pathway of melatonin biotransformation. *Free Radic. Biol. Med.* **29,** 1177–1185
58. Hardeland, R., Pandi-Perumal, S. R., and Cardinali, D. P. (2005) Melatonin. *Int. J. Biochem. Cell Biol.* DOI:10.1016/jbiocel.2005.1008.1020
59. Tesoriere, L., Avellone, G., Ceraulo, L., D'Arpa, D., Allegra, M., and Livrea, M. A. (2001) Oxidation of melatonin by oxoferryl hemoglobin: a mechanistic study. *Free Radic. Res.* **35,** 633–642
60. Ximenes, V. F., de, O. S. S., Rodrigues, M. R., Catalani, L. H., Maghzal, G. J., Kettle, A. J., and Campa, A. (2005) Superoxide-dependent oxidation of melatonin by myeloperoxidase. *J. Biol. Chem.*
61. Horstman, J. A., Wrona, M. Z., and Dryhurst, G. (2002) Further insights into the reaction of melatonin with hydroxyl radical. *Bioorg. Chem.* **30,** 371–382
62. Dellegar, S. M., Murphy, S. A., Bourne, A. E., DiCesare, J. C., and Purser, G. H. (1999) Identification of the factors affecting the rate of deactivation of hypochlorous acid by melatonin. *Biochem. Biophys. Res. Commun.* **257,** 431–439
63. Wong, P. S., Yoshioka, K., Xie, F., and Kissinger, P. T. (1999) In vivo microdialysis/liquid chromatography/tandem mass spectrometry for the on-line monitoring of melatonin in rat. *Rapid Commun. Mass Spectrom.* **13,** 407–411
64. Vakkuri, O., Tervo, J., Luttinen, R., Ruotsalainen, H., Rahkamaa, E., and Leppaluoto, J. (1987) A cyclic isomer of 2-hydroxymelatonin: a novel metabolite of melatonin. *Endocrinology* **120,** 2453–2459
65. Stasica, P., Paneth, P., and Rosiak, J. M. (2000) Hydroxyl radical reaction with melatonin molecule: a computational study. *J. Pineal Res.* **29,** 125–127
66. Lisby, S., Gniadecki, R., and Wulf, H. C. (2005) UV-induced DNA damage in human keratinocytes: quantitation and correlation with long-term survival. *Exp. Dermatol.* **14,** 349–355
67. Schwarz, A., Stander, S., Berneburg, M., Bohm, M., Kulms, D., van Steeg, H., Grosse-Heitmeyer, K., Krutmann, J., and Schwarz, T. (2002) Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. *Nat. Cell Biol.* **4,** 26–31
68. Krutmann, J. (2001) The role of UVA rays in skin aging. *Eur. J. Dermatol.* **11,** 170–171
69. Costa, E. J., Lopes, R. H., and Lamy-Freund, M. T. (1995) Permeability of pure lipid bilayers to melatonin. *J. Pineal Res.* **19,** 123–126
70. Nickel, A., and Wohlrab, W. (2000) Melatonin protects human keratinocytes from UVB irradiation by light absorption. *Arch. Dermatol. Res.* **292,** 366–368
71. Tan, D. X., Manchester, L. C., Reiter, R. J., Qi, W., Hanes, M. A., and Farley, N. J. (1999) High physiological levels of melatonin in the bile of mammals. *Life Sci.* **65,** 2523–2529
72. Skinner, D. C., and Malpaux, B. (1999) High melatonin concentrations in third ventricular cerebrospinal fluid are not due to Galen vein blood recirculating through the choroid plexus. *Endocrinology* **140,** 4399–4405
73. Conti, A., Conconi, S., Hertens, E., Skwarlo-Sonta, K., Markowska, M., and Maestroni, J. M. (2000) Evidence for melatonin synthesis in mouse and human bone marrow cells. *J. Pineal Res.* **28,** 193–202
74. Bubenik, G. A., Hacker, R. R., Brown, G. M., and Bartos, L. (1999) Melatonin concentrations in the luminal fluid, mucosa, and muscularis of the bovine and porcine gastrointestinal tract. *J. Pineal Res.* **26,** 56–63
75. Finocchiaro, L. M., Nahmod, V. E., and Launay, J. M. (1991) Melatonin biosynthesis and metabolism in peripheral blood mononuclear leucocytes. *Biochem. J.* **280(Pt 3),** 727–731

76. Iuvone, P. M., Tosini, G., Pozdeyev, N., Haque, R., Klein, D. C., and Chaurasia, S. S. (2005) Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. *Prog. Retin. Eye Res.* **24,** 433–456
77. Reiter, R. J., and Tan, D. X. (2003) What constitutes a physiological concentration of melatonin? *J. Pineal Res.* **34,** 79–80
78. Slominski, A., Chassalevris, N., Mazurkiewicz, J., Maurer, M., and Paus, R. (1994) Murine skin as a target for melatonin bioregulation. *Exp. Dermatol.* **3,** 45–50
79. Kobayashi, H., Kromminga, A., Dunlop, T. W., Tychsen, B., Conrad, F., Suzuki, N., Memezawa, A., Bettermann, A., Aiba, S., Carlberg, C., and Paus, R. (2005) A role of melatonin in neuroectodermal-mesodermal interactions: the hair follicle synthesizes melatonin and expresses functional melatonin receptors. *FASEB J.* **19,** 1710–1712

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Constitutive and UV-induced metabolism of melatonin in keratinocytes and cell-free systems

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SPECIFIC AIMS

Melatonin is a strong radical scavenger, able to protect the skin against oxidative damage and cell death induced by UV radiation (UVR). So far, the influence of UVB, the most damaging wavelength of UVR, on the molecule melatonin itself has not been investigated. We studied the formation of melatonin metabolites after exposure of melatonin to UVB, determined metabolite identity, and evaluated metabolism determinants in a pure cell-free system and in HaCaT keratinocytes.

PRINCIPAL FINDINGS

1. Identification of photoproducts of melatonin in a cell-free system: 6-hydroxymelatonin, 2-hydroxymelatonin, 4-hydroxymelatonin, and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK)

Irradiation of melatonin solution in a cell-free system with UV-doses at 25, 50, and 100 mJ/cm^2 generated four compounds detected by HPLC with retention times of 30 min (product 1), 34 min (product 2), 35 min (product 3), and 43 min (product 4). The relative amount of each product increased directly proportional with UV-doses **(Fig. 1)**. Product yield was also dependent on the preincubation concentration of melatonin, which was higher with melatonin at 10–3 M than at 10–6 M. Analysis of UV absorption spectra, HPLC retention time, and mass spectrometry of melatonin metabolites identified product 1 as 6-hydroxymelatonin, product 2 as 2-hydroxymelatonin, product 3 as 4-hydroxymelatonin, and product 4 as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK).

2. Determinants of post irradiation melatonin metabolism in keratinocyte supernatants

After irradiation with UVR (25, 50, or 75 mJ/cm^2), supernatants of keratinocytes containing melatonin (10–3 M) produced identical compounds compared with the cell-free system. The metabolites identified in supernatants were products 2 (2-hydroxymelatonin), 3 (4-hydroxymelatonin), and 4 (AFMK), whereas product 1 (6-hydroxymelatonin) was not detected. A linear dose response to increasing UV-doses was observed, as well as a time-dependent increase in melatonin photoproducts. High levels of 2-hydroxymelatonin were detected as early as 40 min after UV exposure and remained high at 190 and 370 min post UV-exposure. Product 3, although generally lower than 2-hydroxymelatonin, increased steadily from 40 min to 370 min post-UVR exposure. AFMK also showed a time-dependent increase with highest levels reached at 40 min, remaining almost unchanged at the collection time point of 190 min to decrease at 370 min. As in the cell-free system, the overall levels of AFMK were higher than those of 2-hydroxymelatonin and 4-hydroxymelatonin.

3. Extra- and intracellular partition of melatonin metabolites

The metabolites 2-hydroxymelatonin, 4-hydroxymelatonin, and AFMK were identified at different proportions in supernatants and lysates of keratinocytes. 2-Hydroxymelatonin was present at higher levels in the extracellular compartment (supernatants) of keratinocytes, where it displayed strong increase after irradiation (50 mJ/cm^2). Intracellularly, 2-hydroxymelatonin was detectable at very low levels, while still showing an increase after UVR exposure. The metabolite 4-hydroxymelatonin was not detected intracellularly, neither under basal condition (without UV irradiation) nor after UV exposure, but in supernatants preincubated with melatonin and exposed to UVR. AFMK was clearly detected in supernatants of irradiated samples (50 mJ/cm^2) where it was 100-fold higher than in nonirradiated samples **(Fig. 2*A*, *upper left inset*)**. Lysates of cells preincubated with melatonin showed that,

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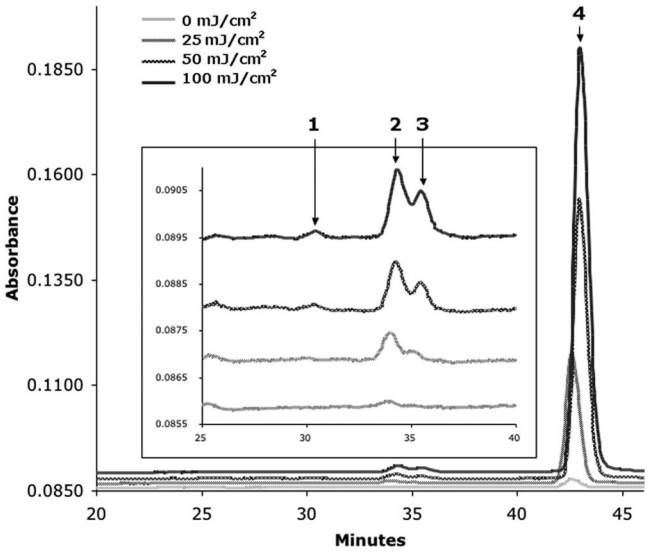

Figure 1. Detection of melatonin metabolites: 6-hydroxymelatonin (product 1), 2-hydroxymelatonin (product 2), 4-hydroxymelatonin (product 3) and AFMK (product 4). Product levels increased after irradiation of melatonin solution (10–3 M) in direct proportion to UV-doses. Levels are highest for AFMK followed by 2-hydroxymelatonin.

under nonirradiated conditions, AFMK levels were low but increased after UVR (3.5-fold) (Fig. 2*A*, *left*). AFMK was also detected in supernatants of samples not preincubated with melatonin (Fig. 2*A*, *upper right inset*) and UV-dependend increase was observed (13-fold). AFMK was also detected intracellularly in samples not preincubated with melatonin and showed again a distinct UV-induced 1.9-fold increase (Fig. 2*A*, *right*).

Melatonin, the main substrate for AFMK production, was expectedly detected at high levels in supernatants of samples preincubated with melatonin (10–3 M) with slight reduction after UV exposure (to 99.7% of levels in the nonirradiated melatonin solution) (Fig. 2*B*, *inset*). Melatonin was not found in cell supernatants without melatonin preincubation, while cell lysates showed detectable levels of melatonin (Fig. 2*B*, *right*). Cell lysates from cells preincubated with melatonin showed expectedly higher melatonin levels than lysates from cells not preincubated with melatonin (Fig. 2*B*, *left*). Nevertheless, with or without melatonin preincubation, cell lysates showed a decrease in melatonin levels after UV exposure (51.4 and 18.9% of unirradiated control, respectively), reciprocal to the increase of AFMK. Under basal conditions (without UV exposure), the ratio of intra- to extracellular melatonin levels in the samples preincubated with melatonin was ~1:800 (0.125%).

4. Kinetics of melatonin metabolism in keratinocytes

Studies in keratinocytes not preincubated with melatonin showed that melatonin and its metabolites, AFMK and 2-hydroxymelatonin, were detected dynamically over 24 h, indicating intense melatonin metabolism. The absolute concentration of melatonin in keratinocytes was 146.0 pmoles/1000 cells, which decreased to 65.0 pmoles/1000 cells after 24 h. AFMK was detected at 17.4 pmoles/1000 cells and showed an increase to 33.6 pmoles/1000 cells after 24 h. The intracellular concentration of 2-hydroxymelatonin increased at 24 h, although to levels lower than AFMK or melatonin (7.8 pmoles/1000 cells that increased to 20.4 pmoles/1000 cells after 24 h). The metabolite 6-hydroxymelatonin was also detected (53.4 pmoles/1000 cells) but showed a decrease after 24 h (16.8 pmoles/1000 cells).

CONCLUSIONS AND SIGNIFICANCE

The present study provides evidence for intense metabolism of melatonin under the influence of UVR in skin

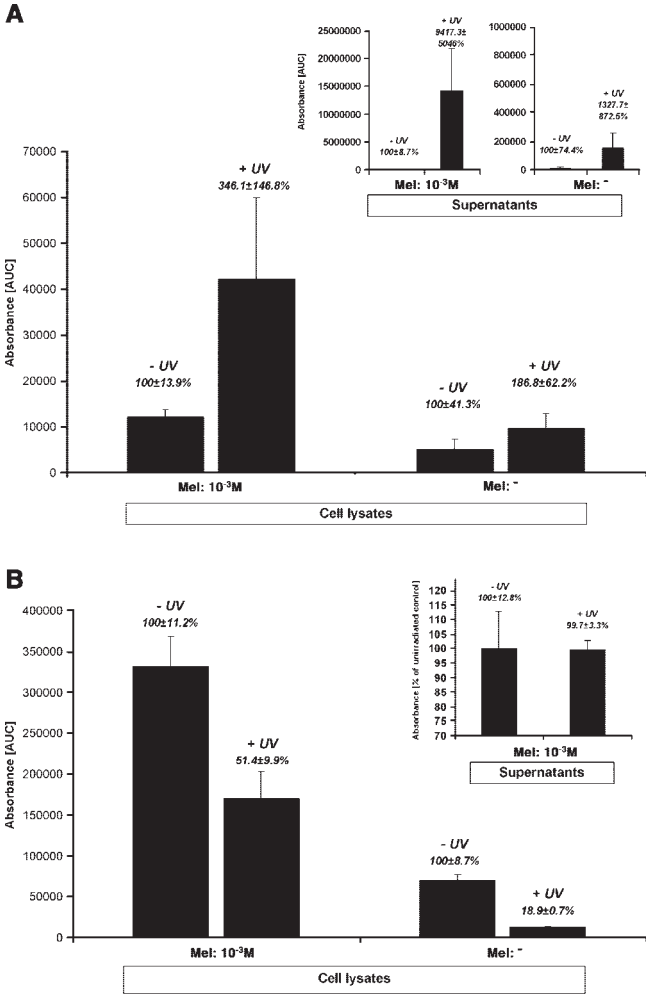

Figure 2. Detection of extra- and intracellular AFMK (*A*) and melatonin (*B*). Levels of AFMK were higher in keratinocytes preincubated with melatonin than in control cells (no melatonin added) (*A*). AFMK was also higher in supernatants that had been preincubated with melatonin, than without melatonin preincubation (*A*, insets). In both conditions and compartments, levels of AFMK increased. Melatonin, in contrast, showed reduced intracellular levels after UV exposure (*B*), whereas extracellular melatonin levels in the condition of melatonin preincubation showed no reduction (*B*, inset). Columns represent means of absolute AUC-values of absorbance peaks from two experiments. Values above columns represent percentage mean ± SEM of unirradiated control.

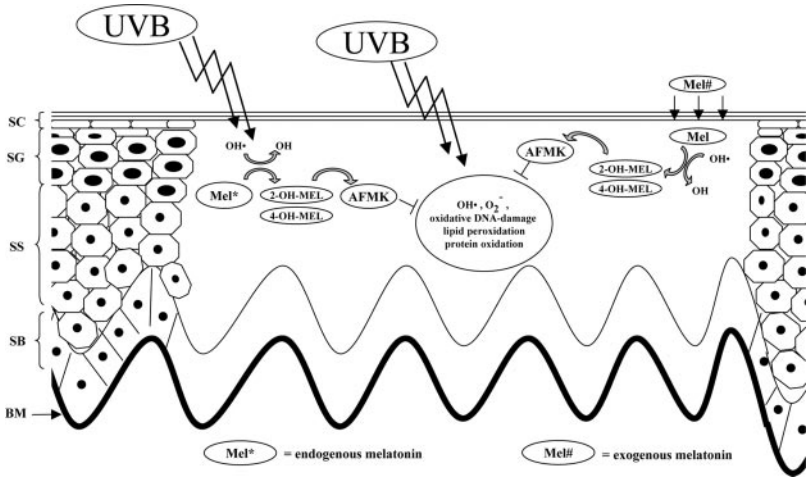

Figure 3. The melatoninergic antioxidative system (MAS) of the skin. Parallel to scavenging UVB-induced reactive oxygen species (ROS) namely hydroxyl radicals, melatonin is also transformed to 2-hydroxymelatonin, 4-hydroxymelatonin, and consecutively to AFMK. AFMK is a potent free radical scavenger and, therefore, by itself capable of protecting the skin against lipid peroxidation, protein oxidation, and oxidative DNA damage. The endogenous melatoninergic system can be supported by exogenous application of melatonin, which penetrates easily through the stratum corneum into deeper layers of the skin. SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale; BM, basement membrane.

cells and in cell-free conditions with generation of 6-hydroxymelatonin, 2-hydroxymelatonin, 4-hydroxymelatonin and AFMK and also shows that melatonin can be produced endogenously in untreated keratinocytes. 6-hydroxymelatonin is the chief product of circulating (endogenous) melatonin in humans and the main metabolite of exogenous melatonin (oral intake). In our study, 6-hydroxymelatonin was detected only in non UV-exposed keratinocytes, which is a fact most likely less relevant in cutaneous biology. The second product, AFMK, is generated by enzymatic and nonenzymatic metabolism, both in vitro and in vivo. AFMK generation has been linked to melatonin oxidization by reactive oxygen species (ROS), which are produced at high levels after exposure to UVR. The major and most damaging ROS, the hydroxyl radical, can be scavenged by melatonin, which is consecutively transformed to an indolyl cation radical and, in the presence of O_2^-, to AFMK. We identified AFMK after irradiation of melatonin in a cell-free system and in supernatants and cell lysates of keratinocytes. The most likely mechanism of AFMK production following UV-exposure is photoinduced cleavage of the pyrrol ring and oxidization by ROS, namely the hydroxyl radical. The third metabolite was 2-hydroxymelatonin, previously identified only in Fenton-type OH-generating systems or in reaction with hypochloric acid. 2-Hydroxymelatonin is the product of a chemical reaction induced by UVR-generated oxygen-based radicals or by the combination of ROS with enzymes such as cytochrome *c*. The fourth product was identified as 4-hydroxymelatonin, a hydroxylated form of melatonin closely related to 2-hydroxymelatonin.

Kinetic studies on the UVR-induced generation of photoproducts of melatonin in cell supernatants clearly showed progressive increase over time (6 h). The increase of 2-hydroxymelatonin and 4-hydroxymelatonin may be explained by oxidation of melatonin suspended in supernatants and simultaneous metabolism of intracellular melatonin with extracellular release. AFMK showed a late decrease between 3 and 6 h after UVR exposure, which may be related to additional metabolism of AFMK to AMK by arylamine formamidase.

The UVR-induced production of 2-hydroxymelatonin and AFMK in cell lysates of keratinocytes is reported for the first time, together with reduction of melatonin, indicative of substrate consumption during UV irradiation. Over a time period of 24 h, an UVR-independent consumption of melatonin was observed and, in turn, an increase of AFMK and 2-hydroxymelatonin.

We used an UV source emitting UVB (280–320 nm; ~60%) and UVA (320–400 nm; ~30%), wavelengths that both occur naturally and are, therefore, relevant in cutaneous biology. UVB causes harmful effects in the epidermis, represented by lipid peroxidataion, protein oxidation, and direct DNA damage in proliferating keratinocytes, whereas UVA induces skin aging in the dermis. These processes are the major target for the protective effects of melatonin. AFMK is known to be a strong radical scavenger that protects against these processes. The increased formation of AFMK under progressively higher doses of UVR would, therefore, support the use of melatonin in topically applied sun protectants since increase in the damaging effect of UVR would reciprocably be accompanied by increase of AFMK production. As a result, the organ could remain in equilibrium between damaging effects of UVR and the protective effects of AFMK.

Both the presence of an enzymatic melatoninergic and functionally active system in the skin and the detection of melatonin levels in human and murine hair follicles support the present findings. We have also shown recently that melatonin, at the same concentration as used in the present study (10–3 M), prevents UVR-induced damage in skin cells.

To conclude, metabolism of melatonin is present in keratinocytes. This metabolism is accompanied by a parallel increase of 2-hydroxymelatonin and AFMK. Most importantly, this process can be independently activated by UVR. Our study supports a novel role of melatonin as protector for the skin against solar radiation. Moreover, the UV-induced production of melatonin metabolites, which are strong antioxidants themselves, defines a novel melatoninergic antioxidative system (MAS) of the skin (**Fig. 3**). Finally, the combination of endogenous melatonin with externally applied melatonin may successfully counteract the multiple processes of skin damage induced by UVR. FJ