Article

Anti-Wrinkle Mechanism of Melatonin in UVB Treated HaCaT Keratinocytes and Hairless Mouse via Inhibition of ROS and Sonic Hedgehog Mediated Inflammatory Proteins

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Abstract: Though melatonin is known to improve ultraviolet B (UVB)-induced oxidative damage and inflammatory conditions via blockade of nuclear factor (NF)-κB, interleukin (IL)-6, there is no report on anti-wrinkle effect of melatonin to date. Hence in the present study, anti-wrinkle mechanism of melatonin was elucidated in UVB treated HaCaT keratinocytes and hairless mice. Herein melatonin protected against a radical initiator tert-Butyl hydroperoxide (t-BOOH) induced reactive oxygen species (ROS) production, matrix metalloprotease 1 (MMP-1) and cytotoxicity in HaCaT keratinocytes. Also, melatonin suppressed the expression of sonic hedgehog (SHH) and GLI for hedgehog signaling, p-NF-κB, cyclooxygenase (COX-2), p-ERK for inflammatory responses in UVB treated HaCaT keratinocytes. Furthermore, melatonin protected skin from wrinkle formation, transdermal water loss in hairless mice irradiated by UVB for 8 weeks. Notably, melatonin prevented against epidermal thickness and dermal collagen degradation in UVB irradiated hairless mice by Hematoxylin & Eosin and Masson’s trichrome staining. Taken together, these findings suggest that melatonin reduces wrinkle formation via inhibition of ROS/SHH and inflammatory proteins such as NF-κB/COX-2/ERK/MMP1.

Keywords: Melatonin, UVB, HaCaT keratinocytes, collagen, ROS, Hedgehog, Cox-2, MMP-1

1. Introduction

Facial wrinkles are known one of the most prominent features for skin aging [1]. Skin aging is usually classified into intrinsic (chronological) and extrinsic (photoaging) aging. Extrinsic aging shows photoaged skin with roughly wrinkles mainly due to the exposure to sunlight including ultraviolet B, while intrinsic aging is induced by the aging genetic factors with the pale and smoothly wrinkle formation [2]. It is well documented that the loss of skin elasticity and degradation of elastic fibers and collagen are usually shown in aged or wrinkled skin [3, 4]. Previous evidences reveal that reactive oxygen species (ROS) in dermal fibroblast and epidermal keratinocyte produce matrix metalloproteinases (MMPs) to induce collagen degradation leading to skin aging, since MMPs degrade the extracellular matrix, including collagen fibers, and thus contributes to wrinkle formation[5]. In particular, UVB exposure upregulates the expression of MMP-1, -3, and -9 in human epidermis to induce wrinkle formation [6].

Thus, recently many phytochemicals such as magnolol [7], abietic acid [8], Galla Chnensis extract [9] and green tea polyphenols [10] were reported to block skin damage due to chronic UVB irradiation. Also, melatonin was reported to protect human keratinocytes from UVB-induced oxidative stress and DNA damage. Nevertheless, the anti-wrinkle mechanism of melatonin still
remains unclear to date. Thus, in the present study, anti-wrinkle molecular mechanism of melatonin was investigated in t-BOOH or UVB treated human keratinocytes and UVB irradiated SKH-1 hairless mice.

2. Results

2.1. Melatonin protected against t-BOOH induced ROS production and cytotoxicity in HaCaT keratinocytes

It was well known that tert-Butyl hydroperoxide (t-BOOH) has been used as a radical polymerization initiator to induce cell damage[11]. To find out protective effect of melatonin (Fig. 1A) in t-BOOH induced cytotoxicity in HaCaT keratinocytes, MTT assay was conducted. As shown in Figure 1b, c, t-BOOH and UVB (30 mJ/cm²) exerted cytotoxicity better than melatonin in HaCaT keratinocytes. However, melatonin significantly suppressed t-BOOH induced cytotoxicity (Figure 2a) and ROS production at the concentrations of 1 and 2 mM in HaCaT keratinocytes (Figure 2b).

Figure 1. Effect of Melatonin, t-BOOH and UVB on the cytotoxicity in HaCaT cells. (a) Chemical structure of melatonin. Molecular weight = 232.2 c (b, c) HaCaT cells were seeded onto 96-well microplates at a density of 1X10⁴ cells/well and treated with various concentrations of Melatonin (0, 0.3, 0.6, 1.25, 2.5 or 5 mM) or t-BOOH (0, 0.15, 0.3, 0.6, 1.25, 2.5 or 5 mM) for 24 h. HaCaT cells were exposed to UVB (30 mJ/cm²) for 15 min, 30 min, 60 min or 120 min. Data represent means ± SD. *p<0.05, ** P<0.01 and *** P<0.001 versus untreated control.

Figure 2. Melatonin suppressed t-BOOH induced cytotoxicity and ROS production in HaCaT keratinocytes. (a) Cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for 6h. (b) ROS generation (%) was measured using ROS-sensitive fluorometric probe 2,7-dichlororfluorescein diacetate (DCFDA) by flow cytometric analysis. Data represent mean ± SD. ** p<0.01 versus treated t-BOOH, ## p<0.001 versus untreated Control.
2.2. Melatonin attenuated the mRNA expression of MMP-1 in t-BOOH or UVB induced HaCaT keratinocytes

There are accumulating evidences that the expression of MMPs was upregulated in t-BOOH induced HaCaT keratinocytes [8, 12]. Also, UVB-induced photoageing is known to be mediated by increased expression of MMP-1 and collagen degradation [13, 14]. To determine the effects of t-BOOH and UVB on MMP-1 expression, qRT-PCR assay was performed. As shown in Figure 3a, melatonin reduced the mRNA expression of MMP-1 in t-BOOH treated HaCaT keratinocytes. Also, melatonin suppressed the mRNA expression of MMP-1 and increased the expression of pro-collagen in UVB treated HaCaT keratinocytes (Figure 3b).

Figure 3. Melatonin attenuated the mRNA expression of MMP-1 and pro-collagen in t-BOOH and UVB induced HaCaT keratinocytes. (a) Cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for 6h. Data represent mean ± SD. *** p<0.001 and ** p<0.01 versus treated t-BOOH, ### p<0.001 versus untreated Control. (b) Cells were pretreated with melatonin for 24h and then irradiated with UVB (30 mJ/cm²) for 10 min or 20 min. Data represent mean ± SD. *** p<0.001, ** p<0.01 and *p<0.05 versus irradiated UVB, ### p<0.001 and ##p<0.01 versus untreated Control.

2.3. Melatonin effectively suppressed the UVB-induced hedgehog signaling and MMP-1 related proteins in HaCaT keratinocytes

To determine whether melatonin inhibits UVB-induced MMP-1(photoaging marker) related proteins, Western blotting was performed. After the exposure to UVB 30 mJ/cm² for 10 min or 20 min, MMP-1 related proteins (p-ERK, Pp-38, COX-2 and MMP-1) were significantly affected at UVB 30 mJ/cm² for 10 min (Figure 4a). To determine whether the intracellular signal mechanism of melatonin, we examined the effects of melatonin on the hedgehog signaling pathway, since hedgehog signaling [15] (SHH/GLI) plays a key role in inducing MMP-1. Consistently, melatonin blocked UVB-induced SHH and GLI expression at 1 and 2 mM (Figure 4b). Also, Figure. 4B showed that the phosphorylation of NF-κB, ERK and COX-2 was increased by UVB irradiation. Nonetheless, melatonin reduced p-NF-κB, p-ERK and COX-2 in the UVB-treated HaCaT keratinocytes. Also, melatonin suppressed the expression of MMP-1 in t-BOOH or UVB treated HaCaT keratinocytes (Figure 4c).
Figure 4. Melatonin attenuated the expression of SHH, GLI and MMP-1 related proteins in t-BOOH and UVB induced HaCaT keratinocytes. (a) HaCaT cells were exposed to UVB (30 mJ/cm²) for 10 min or 20 min and subjected to Western blotting for p-ERK, PP38, COX-2, MMP-1 and β-actin. (b, c) Cells were pretreated with melatonin for 24h and then irradiated with UVB (30 mJ/cm²) for 10 min and subjected to Western blotting for SHH, GLI (nuclear fraction), p-ERK, COX-2, p-NF-κB, MMP-1, β-actin and Lamin B. Cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for 6h.

2.4. Melatonin prevented against loss of water on the dorsal skin of SHK1 hairless mice exposed to UVB

It was well known that UVB can induce skin damages such as melanogenesis, erythema, epidermal thickness in epidermis, leading to wrinkle formation in dermis[16]. As shown in Figure 5a, melatonin significantly blocked the loss of water on the dorsal skin of SHK-1 hairless mice exposed to UVB at 8 weeks following UVB treatments, while the loss of water was accentuated in UVB alone treated control compared to untreated normal control.

2.5. Melatonin suppressed the degree of wrinkles and epidermal thickness induced by UVB irradiation on the dorsal skin of SHK1 hairless mice

The degrees of wrinkles were observed on the dorsal skin of UVB irradiated SHK-1 hairless mice by SILFLO casting method, while those were reduced in melatonin treated SHK-1 hairless mice under UVB exposure as shown in Figure 5b. Consistently, H&E staining showed epidermal thickness induced by UVB was blocked by melatonin treatment and Masson’s trichrome staining revealed that melatonin treatment maintained blue color for collagen staining compared to UVB alone treated control in the dermis of the skin of UVB irradiated SHK-1 hairless mice as shown in Figure 5c,d.

Figure 5. Melatonin significantly suppresses water loss, wrinkles and collagen fibers in the dorsal skin of SKH 1 hairless mice. (a) Effect of melatonin on water loss in the dorsal skin of SKH 1 hairless mice. N, Normal; C, Control +UVB irradiated; P, Positive control: Retinoic acid 2 mg/kg + UVB; MEL 0.5, Melatonin 0.5 mg/kg +UVB; MEL 2, Melatonin 2 mg/kg +UVB. Data represent mean ± SD. *** p<0.001 and * p<0.05 versus untreated Control. (b) Effect of melatonin on wrinkles in the dorsal skin of SKH 1 hairless mice. Melatonin suppressed the degree of induced by UVB irradiation on the dorsal skin of SHK1 hairless mice. Images of skin replicas were casted on the dorsal skin surface of mouse by using SILFLO. (c) Hairless mouse skin was stained with hematoxylin and eosin (x100). Epidermis thickness was measured under light microscopy. Black arrow (epidermis). (d) Collagen fiber formation was evaluated using Masson’s trichrome staining. Collagen fiber bundles are shown in blue area (Blue arrow) (x100).
3. Discussion

Melatonin (N-acetyl-5-methoxytryptamine) is known to have multi-biological activities to regulate circadian rhythms, and work as a hormone or neurotransmitter, anti-inflammatory agent, and antioxidant. Especially, melatonin suppressed ROS production in rat lens[17] and IL-3 stimulated leukocytes[18] and also enhanced the survival of UVB treated keratinocytes by suppressing apoptosis[19].

Herein, anti-wrinkle mechanism of melatonin was elucidated in t-BOOH or UVB treated HaCaT keratinocytes and UVB treated hairless mice.

Melatonin suppressed ROS production and cytotoxicity in free radical initiator t-BOOH treated HaCaT keratinocytes, implying melatonin protects HaCaT keratinocytes from t-BOOH induced cytotoxicity, since ROS play a pivotal role in skin damage by UVB-irradiation.

UVB-induced photoageing is initiated by production of ROS [20, 21], which activate several receptors for IL-1, keratinocyte growth factor and tumor necrosis factor (TNF)-α [22, 23]. Activation of these receptors induces downstream signaling pathways of mitogen-activated protein kinases (MAPKs) such as p38, JNK and ERK, which leads to an increase of the nuclear transcription such as NF-κB and AP-1[22, 24, 25]. Additionally, activation of transcription factor NF-κB induces the expression of inflammatory cytokines such as COX-2, IL-6 and MMPs [26, 27]. Continuous UVB exposure induces expression of MMPs, which degrades collagen by collagen breakdown as well as inhibition of procollagen synthesis [28, 29]. Interestingly, melatonin suppressed the expression of COX-2, p-ERK, p-NF-κB and MMP-1 and increased the expression of procollagen in UVB induced HaCaT cells, indicating melatonin inhibits inflammatory cytokines and photoaging markers, leading to anti-wrinkle effect in skin.

Accumulating evidences reveal that Hh signalling pathway regulates normal growth but its abnormal activation can promote cancer progression and epidermal development [30-33]. Therefore, to identify the molecular mechanism associated with UVB-induced photoageing, the role of Hedgehog (Hh) signaling was evaluated in UVB treated HaCaT cells. Here, melatonin reduced the expression of UVB-induced SHH as a Hh ligand and GLI as a transcriptional effector of Hh pathway, demonstrating melatonin exerts anti-photoaging effect via inhibited Hh signaling pathway.

It was well documented that UVB induces skin damages leading to wrinkle formation due to collagen degradation in the dermis [34, 35]. Here, melatonin protected skin from wrinkle formation, transdermal water loss in UVB treated hairless mice comparable to positive control retinoic acid, indicating melatonin prevents against wrinkle formation in the dermis possibly via inhibition of collagen degradation. Consistently, Masson’s trichrome staining revealed that melatonin treatment maintained blue color staining for collagen and reduced epidermal thickness compared to UVB alone treated group in the dermis of the skin of UVB irradiated SHK-1 hairless mice, implying protective effect of melatonin on wrinkle formation by collagen degradation.

Collectively, melatonin suppressed ROS production and the expression of MMP-1 in t-BOOH-induced HaCaT keratinocytes. Also, melatonin suppressed the expression of UVB-induced MMP-1 and MMP-1 related proteins (inflammatory proteins) and increased pro-collagen in HaCaT keratinocytes and reduced the expression of hedgehog signaling proteins, protected against epidermal thickness, transdermal water loss, collagen degradation and wrinkle formation on the dorsal skin of UVB treated hairless mice. Overall, these findings demonstrate that melatonin protects against UVB induced oxidative skin damage including wrinkle formation via inhibition of ROS, MMP-1 and hedgehog signaling pathway.

4. Materials and Methods

4.1. Chemicals

Melatonin (N-acetyl-5-methoxytryptamine) (M5250) was purchased from Sigma (St. Louis, MO).
4.2. Cell culture

Human skin keratinocyte cell line HaCaT cells (CRL-2404) were purchased from American Type Culture Collection (ATCC). The cells were grown in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL) and antimycotic (amphotericin B 0.25 μg/mL).

4.3. UVB irradiation

HaCaT cells (70–80% confluency) were treated with melatonin (0, 1 or 2 mM) for 24 h. After washed with phosphate-buffered saline (PBS; pH 7.4), the cells were irradiated with 30 mJ/cm² of UVB (312 nm) for 10 min by CL-1000 Ultraviolet crosslinker.

4.4. Cytotoxicity assay

The cytotoxicity of melatonin was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, HaCaT cells (1×10⁴ cells/well) were seeded onto 96-well culture plate and exposed to various concentrations of melatonin for 24 h. The cells were incubated with MTT (1mg/mL) (Sigma Chemical) for 2 h and then treated with MTT lysis solution overnight. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., USA) at 570 nm. Cell viability was calculated as a percentage of viable cells in melatonin treated group versus untreated control.

4.5. RT-qPCR analysis

RT-qPCR was performed with the LightCycler TM instrument (Roche Applied Sciences, Indianapolis, IN) with following primers, human MMP-1 forward: 5′-CATGACTTTCTGGAATTGG -3′; reverse- 5′-CCTGCAGTTGAACCAGCTAT-3′ (Bioneer, Daejeon, Korea), human pro-collagen forward: 5′-CAGGCAAACCTGGTGAACA -3′; reverse -5′-CTCGCCAGGGAACCTCTCT -3′; human GAPDH-forward 5′-GCACCGTCAAGGCTCTAGAAC-3′; reverse-5′-GGATCTCGCTCCTGGAAGAT -3′ (Bioneer, Daejeon, Korea).

4.6. Western blotting

HaCaT Cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) and UVB (30 mJ/cm²), lyzed in lysis solution (50mM Tris–H Cl, pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM EDTA, 1mM NaVO₃, 1mM NaF, and 1× protease inhibitor cocktail) on ice, and spun down at 14,000× g for 20min at 4°C. The supernatants were collected and quantified for protein concentration by using RC DC protein assay kit (Bio-Rad, Hercules, CA, USA), The proteins samples were separated on 4–12% NuPAGE Bis–Tris gels (Novex, Carlsbad, CA, USA) and transferred to a Hybond ECL transfer membrane for detection with antibodies for p-ERK, Pp-38, COX-2, MMP-1, SHH and GLI (Cell signaling Technology, Beverly, MA, USA) Lamin B (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and β-actin (Sigma, St. Louis, MO, USA).

4.7. Nuclear fraction for GLI

HaCaT cells were pretreated with melatonin for 24h and then exposed to UVB irradiation (30 mJ/cm²). Then nuclear fraction of the cells was prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo, Rockford, IL, USA) based on the manufacturer’s protocol for Western blotting with GLI.

4.8. Measurement of reactive oxygen species (ROS) generation

2,7-Dichlorofluorescein diacetate (DCFH-DA) was used to measure the levels of ROS production. HaCaT cells were pretreated with melatonin for 24h and then with t-BOOH (0.6 mM) for 6h. Then 5 μM DCFH-DA for 30 min at 37°C. Fluorescence intensity was measured by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).
4.9. Animals and treatments

Fifty female SKH-1 mice (6 week old) were purchased from Samtako Biokorea (Samtako Biokorea, Osan, Kyunggido, Republic of Korea) and housed at IK Science cage under consistent condition (temperature: 22 ± 1 °C, humidity: 55 ± 3%, 12 h light/ dark cycles). All experiments were performed in accordance with the guidelines established by Kyung Hee University. Mice were acclimated for one week and randomly divided into 5 groups (n = 10) during eight week experimental period; normal group (N); control group (only UVB treated group; three times a week by UV-1000 (Dongseo Science, Korea)); positive control retinoic acid (Sigma Aldrich, USA) group (2 mg/kg) (B); melatonin 0.5 mg/kg (MEL 0.5); melatonin 1 mg/kg (MEL 1). Melatonin was orally administered to the mice of each group daily for 8 weeks, while saline was administered to Normal and control group and retinoic acid was i.p injected in positive control group three times a week for 8 weeks.

UVB irradiation schedule is as follows:
1) 0 week: 60 mJ/cm² for 200sec, three times a week (1 minimal erythema dose; M.E.D)
2) 1week: 120 mJ/cm² for 400sec, three times a week (2 M.E.D)
3) 2~3weeks: 180 mJ/cm² for 600sec 1week: 120 mJ/cm² for 400sec, three times a week (3 M.E.D)
4) 4~5 weeks: 240 mJ/cm² for 800sec, three times a week (4 M.E.D)

4.10. Observation of skin wrinkle formation

The condition of skin wrinkle formation on the dorsal skin of hairless mice exposed to UVB was observed at 4 weeks and 8 weeks, the replicas were casted on the dorsal skin surface of mouse by using SILFLO (Flexico developments LTD. Tokyo, Japan) and then photographed by camera(Nikon D90, Japan).

4.11. Measurement of skin moisture

After mice were kept in the room under the condition of 21.5±2 °C and relative humidity 40±5% for 30 min. Skin moisture was measured in melatonin treated mice by using Corneometer CM 820 (Courage - Khazake, Koln, Germany).

4.12. Histological analysis

Dorsal skin was obtained from melatonin or retinoic acid treated groups and control group mice by biopsy, fixed in 4% paraformaldehyde, dehydrated in ethanol and then embedded in paraffin. Approximately 10µm-thick sections were deparaffinized and stained with hematoxylin-eosin (H&E) and Masson’s trichrome staining. Stained slides were then photographed using a light microscope (ZEISS Observer D2, Germany).

4.13. Statistical analysis

For statistical analysis of the data, Sigmaplot version 12 software (Systat Software Inc., San jose, CA, USA) was used. All data were expressed as means ± standard deviation (SD). Student t-test was used for comparison of two groups. The statistically significant differences were set at p values of <0.05 between control and melatonin treated groups.
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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

UVB     Ultraviolet B
t-BOOH   tert-Butyl hydroperoxide
ROS     Reactive Oxygen Species
MMP-1   Matrix metalloprotease 1
NF-κB   nuclear factor kappa-light-chain-enhancer of activated B cells

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