Inhibition of Connexin 26/43 and Extracellular-Regulated Kinase Protein Plays a Critical Role in Melatonin Facilitated Gap Junctional Intercellular Communication in Hydrogen Peroxide-Treated HaCaT Keratinocyte Cells

Hyo-Jung Lee,¹ Hyo-Jeong Lee,¹ Eun Jung Sohn,¹ Eun-Ok Lee,¹ Jin-Hyoung Kim,¹ Min-Ho Lee,² and Sung-Hoon Kim¹

¹ College of Oriental Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 131-701, Republic of Korea
² College of Life Sciences and Biotechnology, Kyung Hee University, Yongin 446-701, Republic of Korea

Correspondence should be addressed to Sung-Hoon Kim, sungkim7@khu.ac.kr

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Though melatonin was known to regulate gap junctional intercellular communication (GJIC) in chick astrocytes and mouse hepatocytes, the underlying mechanism by melatonin was not elucidated in hydrogen peroxide- (H₂O₂-) treated HaCaT keratinocyte cells until now. In the current study, though melatonin at 2 mM and hydrogen peroxide (H₂O₂) at 300 μM showed weak cytotoxicity in HaCaT keratinocyte cells, melatonin significantly suppressed the formation of reactive oxygen species (ROS) in H₂O₂-treated HaCaT cells compared to untreated controls. Also, the scrape-loading dye-transfer assay revealed that melatonin enhances the intercellular communication by introducing Lucifer Yellow into H₂O₂-treated cells. Furthermore, melatonin significantly enhanced the expression of connexin 26 (Cx26) and connexin 43 (Cx43) at mRNA and protein levels, but not that of connexin 30 (Cx30) in H₂O₂-treated HaCaT cells. Of note, melatonin attenuated the phosphorylation of extracellular signal-regulated protein kinases (ERKs) more than p38 MAPK or JNK in H₂O₂-treated HaCaT cells. Conversely, ERK inhibitor PD98059 promoted the intercellular communication in H₂O₂-treated HaCaT cells. Furthermore, combined treatment of melatonin (200 μM) and vitamin C (10 μg/mL) significantly reduced ROS production in H₂O₂-treated HaCaT cells. Overall, these findings support the scientific evidences that melatonin facilitates gap junctional intercellular communication in H₂O₂-treated HaCaT keratinocyte cells via inhibition of connexin 26/43 and ERK as a potent chemopreventive agent.

1. Introduction

Gap junctional intercellular communication (GJIC) is an important biological mechanism to maintain homeostasis, growth, differentiation, and development of cells and tissues [1]. Gap junctions are made of two hemichannels, called connexons, and each in turn is composed of six molecules of the membrane-spanning connexin (Cx) protein [2, 3].

The gap junctions of human keratinocytes include primarily Cx43, which is abundantly expressed within interfollicular epidermis, and Cx26, which is codistributed with Cx43 in skin [4]. Several studies showed that the downregulation of Cx43 and phosphorylation of Cxs are involved in the carcinogenesis of the skin [4, 5]. Cx43 is phosphorylated by several protein kinases, such as protein kinase C (PKC), casein kinase 1, and mitogen-activated protein kinase (MAPK) [3, 6–8]. Recent evidence suggests that the carcinogenicity of oxidative stress induced by H₂O₂ is attributable to the inhibition of GJIC [8–10].

Melatonin, an indoleamine (N-acetyl-5 methoxytryptamine), produced especially at night in the pineal gland [11, 12], has antioxidant [13, 14], anti-inflammatory [15, 16], antidepressant [17], and antitumor activities against various cancers [18–20]. Though melatonin was recently shown to regulate GJIC in chick astrocyte [21], mouse hepatocytes [22], and MCF-7 breast cancer cells [23, 24], the underlying molecular mechanism by melatonin via GJIC regulation in human keratinocyte HaCaT cells still remains unclear. Thus, in the present study, the molecular mechanism responsible for GJIC regulation by melatonin was examined in human
keratinocyte HaCaT cells using the MTT assay, scrape-loading assay, RT-PCR, western blotting, and flow cytometric analysis for reactive oxygen species (ROS).

2. Materials and Methods

2.1. Chemicals and Reagents. Melatonin (molecular weight: 232), dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, Lucifer Yellow, Trizol reagent, MMLV, Taq polymerase, vitamin C, and 2,7-dichlorofluorescein diacetate (DCFDA) fluorescence dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers (Cx26, Cx30, and Cx43) were purchased from Cosmogenetech (Seoul, Republic of Korea). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic agent were obtained from Welgene (Daegu, Republic of Korea). Sodium dodecyl sulfate (SDS) was purchased from Amresco (Solon, OH, USA). RC DC protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Dimethylformamide was obtained from Merck KGaA (Darmstadt, Germany). Enhanced chemiluminescence (ECL) detection reagent was purchased from Amersham Pharmacia (Piscataway, NJ, USA). Phospho-JNK, JNK phospho-p38 MAPK, p38 MAPK, and ERK antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Cx26, Cx30, Cx43, and phospho-Cx43 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Melatonin was dissolved in DMSO (2 M stock solution). In all experiments, DMSO concentration was kept below 0.2% (v/v) to remove the cytotoxic effect of solvent DMSO.

2.2. Cell Culture. Human keratinocyte HaCaT cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin.

2.3. Cytotoxicity Assay. The cytotoxicity of melatonin was measured by MTT colorimetric assay. HaCaT cells were seeded onto 96-well microplates at a density of 1 × 10^4 cells per well and treated with various concentrations of melatonin for 24 h. MTT working solution (5 mg/mL in PBS) was added to each well and incubated at 37°C for 3 h. The optical density (OD) was determined at 570 nm using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland). Cell viability was calculated as a percentage of viable cells in melatonin or H2O2-treated group versus untreated control by the following equation: cell viability (%) = [OD (melatonin) – OD (blank)]/[OD(Control) – OD (blank)] × 100.

2.4. Scrape-Loading Dye-Transfer Assay. GJIC of the cells was assessed by the scrape-loading dye-transfer (SLDT) technique described by EL-Fouly et al. [25] with some modifications. HaCaT cells (cell confluency: 80–90%) incubated in 35 mm dishes for 24 h were treated with H2O2 (300 μM) or melatonin (1 or 2 mM), respectively. Following incubation, the cells were washed twice with 2 mL of PBS. Lucifer Yellow was added to the washed cells, and three scrapes were made with a surgical steel-bladed scalpel at low-light intensities. Three scrapes were performed to ensure that the scrape traversed a large group of confluent cells. After 3 min incubation, the cells were washed with 10 mL of PBS and then fixed with 2 mL of a 4% formalin solution. The distance traveled by the dye in a direction perpendicular to the scrape was observed with an inverted Axio Axiolab S 100 fluorescent microscope (Carl Zeiss).

2.5. Total RNA Isolation and RT-PCR Analysis. Total RNA was prepared by using Trizol reagent according to the manufacturer's instructions. Total RNA (1.0 μg) was reverse transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA) by incubation at 25°C for 10 min, at 42°C for 30 min, and at 99°C for 5 min. The synthesized cDNA was amplified using TaKaRa Taq DNA polymerase (TaKaRa Biotechnology, Shiga, Japan) and the following specific primers: Cx26 (sense 5'-TCTTTTCTTCCAGACAGCAAACGC-3'; antisense 5'-CTGGAATGGAATACCTGG-3'), Cx30 (sense 5'-CGACGCATCCCTTTTGCGGACT-3'; antisense 5'-ATGCTCCTTTTGTCAAGACGT-3'), Cx43 (sense 5'-TACCATGCGGACACGTGTCCGCT-3'; antisense 5'-GAATTCTGGTTATCTACGGGAA-3'), and GAPDH (sense 5'-GTGGATATGTTGCTCATCA-3', antisense 5'-AAGACACACCTCAGG-3'). PCR products were run on 2% agarose gel and then stained with ethidium bromide (EtBr).

2.6. Measurement of Reactive Oxygen Species (ROS) Production. ROS level was measured using 2,7-dichlorofluorescein diacetate (DCFDA) fluorescence dye. Cells were incubated with 1 μM DCFDA at 37°C for 30 min. Fluorescence intensity was measured by BD FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

2.7. Western Blotting. Cells (1 × 10^6 cells/mL) were treated with various concentrations of melatonin (0, 1, or 2 mM) for 24 h, lyzed in lysis (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and 1x protease inhibitor cocktail) on ice, and spun down at 14,000 × g for 20 min at 4°C. The supernatants were collected and quantified for protein concentration by using RC DC protein assay kits (Bio-Rad, Hercules, CA, USA). The protein 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 Cx26, Cx30, Cx43 and phospho-Cx43 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), phospho-JNK, JNK, phospho-p38 MAPK, p38 MAPK, phospho-ERK, and ERK (Cell signaling Technology, Beverly, MA, USA), and β-actin (Sigma, St. Louis, MO, USA).

2.8. Statistical Analyses. All data were expressed as means ± SD. The statistically significant differences between control and melatonin-treated groups were calculated by ANOVA test followed by a post hoc analysis (Tukey or Dunnett's
3. Results

3.1. Melatonin and H$_2$O$_2$ Exerted Weak Cytotoxicity in HaCaT Cells. To determine nontoxic concentrations of melatonin and H$_2$O$_2$, the cytotoxic effects of melatonin and H$_2$O$_2$ were evaluated in HaCaT cells by MTT assay. Cells were exposed to various concentrations of melatonin (0, 0.25, 0.5, 1, 2, or 4 mM) and H$_2$O$_2$ (0, 150, 300, or 600 μM) for 24 h, and then MTT assay was performed. As shown in Figures 1(b) and 1(c), melatonin and H$_2$O$_2$ showed weak cytotoxic effect in HaCaT cells. Thus, a concentration of 300 μM H$_2$O$_2$ was used for all experiments.

3.2. Melatonin Reduced ROS Production and Facilitated the Decreased GJIC Activity in H$_2$O$_2$-Treated HaCaT Cells. H$_2$O$_2$ is well known to produce free radicals to inhibit gap junctional intercellular communication [26]. As shown in Figure 2(a), melatonin reduced ROS production to 5.83% compared to H$_2$O$_2$-treated control (22%) in HaCaT cells. Consistently, melatonin enhanced intercellular communication disturbed by H$_2$O$_2$ in HaCaT cells by scrape-loading dye-transfer assay as shown in Figures 2(c) and 2(d).

3.3. Melatonin Significantly Enhanced the Expression of Cx26 and Cx43 at mRNA and Protein Levels, but Not That of Cx30 in H$_2$O$_2$-Treated HaCaT Cells. The phosphorylation of the gap junction protein Cx43 is directly associated to functional GJIC [27]. To investigate the effect of melatonin on connexins at mRNA and protein levels in H$_2$O$_2$-treated HaCaT cells, RT-PCR and western blot analyses were carried out. As shown in Figures 3(a) and 3(b), mRNA level of Cx26 and Cx43 were reduced by H$_2$O$_2$-alone treatment, while melatonin enhanced the mRNA level of them in H$_2$O$_2$-treated HaCaT cells. mRNA level of Cx30 did not change in H$_2$O$_2$- or melatonin-treated cells. Consistently, melatonin increased the protein level of Cx26 and Cx43 in H$_2$O$_2$-treated HaCaT cells (Figures 3(d) and 3(e)). We also observed that melatonin suppressed the phosphorylation of Cx43 in H$_2$O$_2$-treated HaCaT cells (Figure 3(c)).
3.4. Melatonin Significantly Decreased the Phosphorylation of ERK Alone, but Not p38 MAPK or JNK in H2O2-Treated HaCaT Cells. The effect of melatonin on MAPK signaling was investigated in H2O2-treated HaCaT cells. Melatonin attenuated the phosphorylation of ERK, but did not significantly affect that of p38 MAPK and JNK in H2O2-treated HaCaT cells, while H2O2 activated the phosphorylation of ERK, p38, and JNK proteins as shown in Figures 4(a) and 4(b). Next, in order to confirm that the GJIC by H2O2 is mediated by ERK pathway, we used the ERK inhibitor PD98059. As shown in Figures 4(c) and 4(d), ERK inhibitor PD98059 effectively recovered the decreased activity of GJIC in H2O2-treated HaCaT cells.

3.5. Combined Treatment of Melatonin and Vitamin C at Low Concentrations Exerted the Synergy in Reducing ROS Production in H2O2-Treated HaCaT Cells. In order to evaluate the synergistic effect of melatonin with other antioxidant, we used vitamin C. As shown in Figure 5(a), melatonin (200 μM) or vitamin C (10 μg/mL) alone at low concentration did not affect Cx34 in H2O2-treated HaCaT cells. In contrast, combined treatment of melatonin and vitamin C promoted the expression of Cx34. Similarly, though melatonin at 2 mM suppressed ROS generation induced by H2O2, low concentration (200 μM) of melatonin did not affect ROS production as in Figure 5(b). As shown in Figure 5(b), melatonin (200 μM) or vitamin C (10 μg) alone did not affect
Figure 3: Melatonin significantly enhanced the expression of Cx26 and Cx43 at mRNA and protein levels, but not that of Cx30 in H₂O₂-treated HaCaT cells. (a) Cells were exposed to H₂O₂ (300 μM) with or without melatonin (1 or 2 mM) for 24 h. (a) mRNAs expressions of Cx26, Cx30, and Cx43 were analyzed by RT-PCR. Grapes represent relative level of Cx26, Cx30, and Cx43/GAPDH. (b) Quantification of mRNAs expression. Phosphorylation of Cx43 (c) and protein expressions of Cx26, Cx30, and Cx43 (d) in melatonin-H₂O₂-treated cells were analyzed by western blot. (e) Grapes represent relative level of Cx26, Cx30, and Cx43/β-actin.
ROS production, but combination of melatonin and vitamin C significantly reduced ROS production to 16.15% compared to H$_2$O$_2$-treated control (23.56%).

4. Discussion

H$_2$O$_2$ plays an important role in the multistep process of carcinogenesis and directly promotes transformation in many in vivo and in vitro model systems [28–30]. In the present study, melatonin suppressed ROS production and facilitated H$_2$O$_2$-mediated inhibition of GJIC in HaCaT cells, implying the antioxidant and anti-carcinogenic potential of melatonin, which was supported by previous studies that the carcinogenicity of H$_2$O$_2$ is attributable to the inhibition of GJIC [31]. Likewise, antioxidants such as vitamin C and quercetin protect against the disruption of GJIC induced by H$_2$O$_2$ [32].

There are several lines of evidences that malignant lesions reveal abnormal expression of connexins and decreased GJIC [33–35]. The function of GJIC can be modulated at the
multi-stages during the turnover of connexins by transcriptional, translational, and posttranscriptional mechanisms. Hence, prevention or inhibition of decreased GJIC can be an important target for cancer therapy. As suggested, H$_2$O$_2$ induced downregulation of connexins, thereby disrupting the GJIC system [5]. Here we found that melatonin recovered the reduced phosphorylation of Cx26 and Cx43 induced by H$_2$O$_2$ at protein and mRNA levels, but not that of Cx30 in H$_2$O$_2$-treated HaCaT cells, indicating that melatonin regulates GJIC via activation of Cx26 and Cx43 signaling.

MAPKs are considered to play important roles in GJIC [36]. Also, ROS-activated MAPK cascades phosphorylate the various proteins involved in cell growth and development [37]. Previous studies revealed that H$_2$O$_2$-dependent ERK and p38 kinase activation lead to depressed GJIC and enhanced connexin degradation [36]. However, in the
current study, melatonin significantly decreased the phosphorylation of ERK alone, but not p38 MAPK or JNK. Furthermore, ERK inhibitor PD98059 effectively recovered the lowered activity of GJIC in H₂O₂-treated HaCaT cells, suggesting the critical role of ERK in recovering the decreased GJIC activity by H₂O₂. Interestingly, combined treatment of melatonin (200 μM) and vitamin C (10 μg/mL) that do not affect ROS production significantly reduced ROS production in H₂O₂-treated HaCaT cells, implying the synergistic effect of melatonin and vitamin C at low concentrations. However, it is also required to confirm this synergistic effect in small animals or humans in the near future.

In summary, melatonin showed weak cytotoxicity in HaCaT cells, reduced ROS production, recovered the disturbed GJIC, enhanced the expression of Cx26 and Cx43 at mRNA and protein levels, suppressed the phosphorylation of ERK, and enhanced synergy with vitamin C in H₂O₂-treated HaCaT cells (Figure 6). Overall, our findings suggest that melatonin recovers decreased GJIC via enhancement of Cx26 and Cx43 and inhibition of ROS production and ERK phosphorylation.

**Authors’ Contribution**

H.-J. Lee and S.-H. Kim conceived and coordinated the studies, designed the experiments, and drafted the paper. H.-J. Lee, H.-J. Lee, and E. J. Sohn performed experiments and statistical analyses and analyzed data. E.-O. Lee, J.-H. Kim, and M.-H. Lee analyzed data. H.-J. Lee and S.-H. Kim analyzed data and edited the paper. All authors read and approved the final paper. H.-J. Lee and M.-H. Lee are contributed equally to this paper.

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