Enhancement of high glucose-induced PINK1 expression by melatonin stimulates neuronal cell survival: Involvement of MT2/Akt/NF-κB pathway

Xaykham Onphachanh1,2 | Hyun Jik Lee1,3 | Jae Ryong Lim1,3 | Young Hyun Jung1,3 | Jun Sung Kim1,3 | Chang Woo Chae1,3 | Sei-Jung Lee4 | Amr Ahmed Gabr1,5 | Ho Jae Han1,3

Abstract

Hyperglycemia is a representative hallmark and risk factor for diabetes mellitus (DM) and is closely linked to DM-associated neuronal cell death. Previous investigators reported on a genome-wide association study and showed relationships between DM and melatonin receptor (MT), highlighting the role of MT signaling by assessing melatonin in DM. However, the role of MT signaling in DM pathogenesis is unclear. Therefore, we investigated the role of mitophagy regulators in high glucose-induced neuronal cell death and the effect of melatonin against high glucose-induced mitophagy regulators in neuronal cells. In our results, high glucose significantly increased PTEN-induced putative kinase 1 (PINK1) and LC-3B expressions; as well it decreased cytochrome c oxidase subunit 4 expression and Mitotracker™ fluorescence intensity. Silencing of PINK1 induced mitochondrial reactive oxygen species (ROS) accumulation and mitochondrial membrane potential impairment, increased expressions of cleaved caspases, and increased the number of annexin V-positive cells. In addition, high glucose-stimulated melatonin receptor 1B (MTNR1B) mRNA and PINK1 expressions were reversed by ROS scavenger N-acetyl cysteine pretreatment. Upregulation of PINK1 expression in neuronal cells is suppressed by pretreatment with MT2 receptor-specific inhibitor 4-P-PDOT. We further showed melatonin-stimulated Akt phosphorylation, which was followed by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) phosphorylation and nuclear translocation. Silencing of PINK1 expression abolished melatonin-regulated mitochondrial ROS production, cleaved caspase-3 and caspase-9 expressions, and the number of annexin V-positive cells. In conclusion, we have demonstrated the melatonin stimulates PINK1 expression via an MT2/Akt/NF-κB pathway, and such stimulation is important for the prevention of neuronal cell apoptosis under high glucose conditions.

KEYWORDS

diabetes mellitus, glucose, melatonin, mitophagy, neuronal cell apoptosis, PTEN-induced putative kinase 1
1 | INTRODUCTION

Hyperglycemia is a major hallmark of diabetes mellitus (DM), which leads to diabetic complications including neuronal degeneration.\textsuperscript{1-3} Many reports have shown increases in hippocampal and cortical neuron apoptosis and reductions in hippocampal neuronal volume in diabetic patients.\textsuperscript{4,5} These results suggest that neuronal cell apoptosis is a representative phenomenon indicating the pathological progress of DM-associated neurodegeneration, thus suggesting the need for development of a strategy for suppressing neuronal apoptosis in DM patients with hyperglycemia. In addition, previous researchers have reported that oxidative stress induced by hyperglycemia is an essential pathogenic factor contributing to several DM-associated complications.\textsuperscript{6,7} Recently, several researchers reported a relationship between mitophagy-regulated oxidative stress and the pathogenesis of diabetic heart and diabetic neuropathy, indicating that the interest of researchers in the protective action of mitophagy in DM patients has increased.\textsuperscript{8,9} It has been shown that PTEN-induced putative kinase 1 (PINK1), Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), and NIX are major mitophagy regulators that can trigger mitophagy under oxidative and metabolic stress conditions.\textsuperscript{10-12} Although there have been many investigations into mitophagy regulators under oxidative stress conditions, the role of reactive oxygen species (ROS) in expression and activation of mitophagy regulators is dependent on cell type and the physiological environment.\textsuperscript{11,13,14} A previous study reported that impairment of PINK1-dependent mitophagy is closely related to diabetes susceptibility and proposed that PINK1 is a key factor in DM pathogenesis.\textsuperscript{15} Moreover, some reports have suggested that regulation of mitophagy is a promising therapeutic strategy for protection against both severe oxidative stress in DM and DM-associated complications.\textsuperscript{16-18} However, there are few reports showing the effect of hyperglycemia on mitophagy regulation in neuronal cells. Therefore, the present study investigated details of the signaling pathway regulating mitophagy of neuronal cell under high glucose conditions in order to provide important insights into the prevention of neuronal cell death.

Melatonin (\textit{N}-acetyl-\textit{N}-methoxytryptamine) is produced and secreted from nonneural tissue as well as the pineal gland, which is involved in cellular physiological functions.\textsuperscript{19,20} Melatonin signaling is induced by two types of G-protein-coupled receptors; melatonin receptor 1A (MT\textsubscript{1}, encoded by \textit{MTNR1A}) and melatonin receptor 1B (MT\textsubscript{2}, encoded by \textit{MTNR1B}).\textsuperscript{21} Results of genome-wide association studies have shown a relationship between variation of MT\textsubscript{2} (\textit{MTNR1B}) in pancreatic islet cells and type 2 diabetes mellitus (T2DM) occurrence, highlighting the role of MT\textsubscript{2} in T2DM pathogenesis.\textsuperscript{22,23} Previous data suggested that decreased melatonin signaling through deleterious MT\textsubscript{2} receptor activity increases type 2 diabetes risk.\textsuperscript{24} These results were line with various studies, which showed that high melatonin levels lead to decreased risk of diabetes.\textsuperscript{25,26} However, increased melatonin signaling is a risk factor for T2DM.\textsuperscript{27} Thus far, the mechanisms underlying these discrepancies actions remain elusive. Regardless, it has been determined that melatonin’s antioxidative effect is induced through various mechanisms that regulate its direct and indirect oxidative stress suppressive actions.\textsuperscript{19,20} In addition, the melatonin-activated intracellular antioxidative system is effective in the prevention of neuronal cell apoptosis in neurodegenerative disease models.\textsuperscript{28-30} Several studies have focused on the role of melatonin in mitophagy regulation.\textsuperscript{31-33} In particular, recent reports on the effect of melatonin on PINK1 expression have provided new insights into melatonin application and mitophagy regulation.\textsuperscript{32,34} However, which mitophagy regulators contribute to the mitophagy-induced antioxidative effect of melatonin in DM patients has been incompletely elucidated. In addition, studies into the melatonin-signaling mechanism in mitophagy regulation are required to describe fully melatonin’s protective role.

The SK-N-MC and SH-SY5Y neuroblastoma cell lines and mouse hippocampal neurons have been widely used for investigating the pathogenesis of neuronal diseases related to hyperglycemia and DM.\textsuperscript{35-38} Moreover, use of the neuronal cell lines has advantages in studies into the identification of molecular mechanism due to their high homogeneity and reproducibility. And, identifying and describing in detail the mechanism involved in the signaling pathway controlling mitophagy regulators under high glucose conditions and elucidating the actions of melatonin in neuronal cell models are important for the development of an effective therapeutic strategy for hyperglycemia-induced neuronal cell death. Therefore, we investigated the role of mitophagy regulators mediated by high glucose in neuronal cell death and the regulatory mechanism of melatonin against mitophagy regulators in neuronal cells under high glucose conditions.

2 | MATERIALS AND METHODS

2.1 | Materials

Human neuroblastoma cells of the SK-N-MC and SH-SY5Y cell lines were kindly provided by the Korean Cell Line Bank (Seoul, Korea). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA), while BNIP3, NIX, Mitofusin-2 (Mfn2), p-Akt (Thr308), p-Akt (Ser473), Akt, p-NFkB (Ser536), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p-ERK (Tyr204), ERK, PKC, MT\textsubscript{2}, lamin A/C, β-actin, β-tubulin, MT\textsubscript{1}, and caspase-9 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). PINK1, microtubule-associated proteins 1A/1B light chain 3B (LC-3B), and MT\textsubscript{2} antibodies were
purchased from Novus Biologicals (Littleton, CO, USA). P-pan PKC (βII Ser660) and caspase-3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Cytochrome c oxidase subunit 4 (COX4) antibody was purchased from Abcam (Cambridge, MA, USA). Ubiquitin antibody was purchased from AbFrontier (Seoul, Korea). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were purchased from Thermo Fisher (Waltham, MA, USA). Melatonin (Mel, 5-methoxy-N-acetyltryptamine) was obtained from Sigma Chemical (St. Louis, MO, USA), while d-glucose, l-glucose, N-acetyl cysteine (NAC), LY294002, SN50, 4-P-PDOT, and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA), small interfering RNAs (siRNAs) for PINK1, and nontargeting (NT) siRNAs were purchased from Dharmacon (Lafayette, CO, USA). All reagents used in this study were high-quality commercial preparations.

2.2 Cell culture

The SK-N-MC and SH-SY5Y cells were cultured in 5x10^5 cells/60 mm dish in 3 mL high glucose Dulbecco’s essential medium (DMEM; Gibco, Grand Island, NY, USA) including 10% FBS, 1% antibiotic (penicillin, streptomycin, and fungizone) high glucose Dulbecco’s essential medium (DMEM; Gibco). The cells were incubated at 37°C with 5% CO₂ for 72 hours, which was followed by washing with phosphate-buffered solution (PBS). The growth medium was then changed to serum-free high glucose DMEM for an additional 24 hours. After starvation, cells were plated with high glucose DMEM and reagents.

2.3 Mouse primary hippocampal neuron culture

Mouse hippocampus was isolated from brains of 17-day old mouse embryo with 0.05% trypsin solution. After isolation, dissociated cells were counted by using a hemocytometer and 2x10^5 dissociated cells were plated on poly-d-lysine-coated 35-mm dishes with neurobasal plating medium (neurobasal medium (Gibco) containing B27 supplement (1 mL/50 mL), 25 μmol/L glutamate (Sigma-Aldrich), 0.5 mmol/L glucose (Sigma-Aldrich), 10% horse serum (Gibco), and 1 mmol/L HEPES (Sigma-Aldrich). Cells were incubated at 37°C with 5% CO₂ for 24 hours. After incubation, neuronal cells replaced with same volume of neurobasal feeding medium (neurobasal medium containing B27 supplement [1 mL/50 mL], 0.5 mmol/L, glutamine, 10% horse serum, 1 mmol/L HEPES and 5 μmol/L of cytosine arabinoside [AraC; Sigma-Aldrich]). After incubation of 24 hours, the medium was changed to neurobasal feeding medium without AraC. Neuronal cells were then cultured for 12 days. All primary hippocampal culture protocols followed the National Institutes of Health Guidelines for the Humane Treatment of Animals. This protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-151116-1).

2.4 RNA isolation and reverse transcription polymerase chain reaction

RNA samples were extracted by using a commercial RNA extraction kit (TaKaRa Biomedical, Otsu, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was performed with 1 μg of extracted RNA and a Maxime™ RT-PCR premix kit (iNtRON Biotechnology, Sungnam, Korea) to produce cDNA. RT-PCR was performed for 1 hours at 45°C and 5 minutes at 95°C.

2.5 Real-time polymerase chain reaction

The cDNA sample was amplified with a QuantiSpeed™ SYBR kit (Life Technologies, Gaithersburg, MD, USA). The mRNA expression levels of the PINK1, BNIP3, NIX, MTNR1A, MTNR1B, and ACTB genes were determined by using a Rotor-Gene 6000 real-time thermal cycling system (Corbett Research, Mortlake, NSW, Australia) with mRNA primers and 1 μg of cDNA sample. The primer sequences of mRNAs are described in Table S1. Melting curve analysis was performed by using software provided by Rotor-Gene 6000 Series software (Qiagen, Hilden, Germany) to confirm the identity and specificity of the real-time PCR products. Gene expression level was normalized to the mRNA expression of ACTB.

2.6 Small interfering RNA transfection

Prior to performing small interfering (si) RNA transfection, cells were grown until 70% of the dish surface was covered. PINK1 (cat no. L-004030-00-0005; Dharmacon) -specific or NT (cat no. L-001206-13-20; Dharmacon) siRNAs (20 nmol/L) were transfected into cells for 24 hours along with Turbofect™ transfection reagent (Thermo Fisher) according to the manufacturer’s manual. Subsequently, cells were incubated at 37°C with 5% CO₂ for 24 hours. The siRNAs sequences of PINK1 and NT are described in Table S2.

2.7 Trypan blue exclusion cell viability assay

Cells were washed with cold PBS, followed by incubation with 0.05% trypsin. Dissociated cells were collected and placed on ice. Suspended cells were stained with 0.4% trypan blue (Sigma-Aldrich) to identify dead cells. Stained (dead) and unstained (live) cells were counted by using a Petroff–Hausser counting chamber (Hausser Scientific, Horsham,
PA, USA). Cell viability=\left\{1-(\text{number of trypan blue-stained cells/number of total cells})\right\} \times 100.

2.8 | Nuclear fraction preparation

Harvested cells were lysed in nuclear fraction lysis buffer (1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 8.1 mmol/L Na_2HPO_4, 137 mmol/L NaCl, 1.5 mmol/L KH_2PO_4, 2.7 mmol/L KCl, 2.5 mmol/L EDTA, and 10 mg/mL leupeptin [pH 7.5]) by pipetting and incubated for 10 minutes on ice. Cell lysates were centrifugated at 842 g at 4°C for 5 minutes, and the supernatant was collected as a nonnuclear fraction. The residual pellet was lysed with radio-immunoprecipitation assay (RIPA) lysis buffer on ice for 25 minutes, and subsequently centrifugated at 21 000 g at 4°C for 30 minutes. The supernatant, as the nuclear fraction, was collected.

2.9 | Western blot analysis

Cells were harvested, washed twice with cold PBS, collected by using a cell scraper, and lysed with RIPA buffer (Thermo Fisher) and a proteinase and phosphatase inhibitor (Thermo Fisher) for 30 minutes on ice. The lysates were then cleared via centrifugation (21 000 g at 4°C for 30 minutes), and the supernatant was collected. The protein concentration was determined by using a bicinchoninic acid (BCA) assay kit (Thermo Fisher). Equal amounts of protein (10 μg) were loaded in 8%-15% sodium dodecyl sulfate-polyacrylamide gel sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, and the membrane washed with TBST solution (150 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 7.6], and 0.1% Tween 20). The membrane was blocked with 5% skim milk (Gibco) and 5% bovine serum albumin (Sigma-Aldrich) in TBST solution for 1 hour; subsequently, the membrane was washed with TBST and incubated with the appropriate primary antibody (1:1000) overnight at 4°C. The membrane was then washed and incubated with HRP-conjugated secondary antibody (1:10 000) for 4 hours at 4°C. The western blot bands were visualized by using an enhanced chemiluminescence solution (Bio-Rad, Hercules, CA, USA).

2.10 | Preparation of co-immunoprecipitation sample

Co-immunoprecipitation was performed by using a commercial kit (Thermo Fisher) according to supplier’s manual. Harvested cells were lysed with IP lysis buffer and proteinase inhibitor cocktail (Thermo Fisher). Lysate samples were immunoprecipitated by Mfn2-conjugated resin. Mfn2-interacted protein was acquired by incubation in elution buffer. Sample buffer was added to eluted sample, and boiled at 100°C for 5 minutes.

2.11 | Measurements of intracellular reactive oxygen species levels

Intracellular ROS level determination was performed by using CM-H_2DCF-DA staining (DCF-DA; Life Technologies). Cells were washed twice with PBS and incubated with 0.05% trypsin for 5 minutes. Dissociated cells (5×10^5) were centrifugated at 1497 g for 5 minutes and then incubated in the dark with 10 μmol/L DCF-DA in PBS for 30 minutes at 37°C. Cells were then washed with PBS and 100 μL of cell suspension was loaded in a 96-well black plate, and staining results measured by luminometer (VICTOR3; PerkinElmer, Waltham, MA, USA).

2.12 | Annexin V/PI fluorescence-activated cell sorter analysis

Annexin V and PI double staining was performed by using an annexin V/PI apoptosis detection kit (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer’s manual. After treatment, cells were detached with 0.05% trypsin. Cells were counted 1×10^5 cells, followed by centrifugation at 1497 g for 5 minutes. Cells were then resuspended in the binding buffer supplied with the apoptosis detection kit, and immunostained with 5 μL of fluorescein isothiocyanate (FITC)-conjugated Annexin V and 5 μL of PI for 15 minutes at room temperature. Cell apoptosis was measured by using flow cytometry (Beckman Coulter, Fullerton, CA, USA). Data were analyzed by using CXP software (Beckman Coulter). Annexin V-positive with PI-positive (Q2) or PI-negative cells (Q4) and Annexin V-negative with PI-positive cells (Q1) were considered apoptotic. Annexin V-negative and PI-negative cells (Q3) were considered viable. To determine the percentage of apoptotic cells, the following formula was used: Apoptotic cells=Q1+Q2+Q4.

2.13 | Immunocytochemistry

Cells were cultured in a confocal dish (Thermo Fisher). After treatment, cells were fixed with 80% aceton in PBS for 10 minutes at −20°C. To inhibit nonspecific binding of antibodies, cells were incubated with 5% normal goat serum (Sigma-Aldrich) in PBS. Subsequently, cells were incubated with a 1:100 dilution of primary antibody for 2 hours at room temperature. After washing, cells were incubated with Alexa fluor-conjugated secondary antibody and PI for 1 hour at room temperature. Images were obtained by using a Fluoview 300 confocal microscope (Olympus, Tokyo, Japan). Fluorescence intensity was analyzed by using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).
2.14 | Measurement of mitochondrial ROS generation

To evaluate the generation of mitochondrial ROS, MitoSOX™ mitochondrial superoxide indicator (Thermo Fisher) was used. Cells were washed with PBS and then incubated with 10 μmol/L MitoSOX™ solution in PBS at 37°C for 15 minutes. Cells were again washed with PBS and incubated with 0.05% trypsin for 5 minutes. Cells were then washed twice with PBS, followed by suspending the cells in 500 μL PBS. MitoSOX™-positive cells were detected by using flow cytometry (Beckman Coulter). Cells that had similar side scatter and forward scatter levels were analyzed by using Flowing Software (developed by Perttu Terho, Turku, Finland).

2.15 | Measurement of mitochondrial membrane potential

To measure mitochondrial membrane potential, tetramethylrhodamine ethyl ester (TMRE) (Sigma-Aldrich) was used. After treatment, cells were washed with PBS and incubated with 200 nmol/L of TMRE solution in PBS at 37°C for 30 minutes. Cells were then washed with PBS and detached by using 0.05% trypsin solution. Detached cells were washed twice with PBS and suspended with 500 μL of PBS. TMRE-positive cells were detected by using flow cytometry (Beckman Coulter). Cells that had similar side scatter and forward scatter levels were analyzed by using Flowing Software (developed by Perttu Terho).

2.16 | Statistical analysis

All quantitative data are presented as a mean value±standard error of mean (SEM). Comparisons of treatment means with that of control groups were conducted by using Student’s t test for two group analysis. Differential among experimental groups were analyzed by using ANOVA. A P value<.05 was considered statistically significant.

3 | RESULTS

3.1 | Effect of high glucose on PINK1-mediated mitophagy in neuronal cells

First, we determined the effect of high glucose on mitophagy regulator expression in SK-N-MC cells. As shown in Figure 1A, PINK1 mRNA expression was significantly increased at 25 and 50 mmol/L d-glucose, but BNIP3 and NIX mRNA expressions were not affected by d-glucose treatment. In addition, PINK1 increased and COX4 expressions decreased in a d-glucose dose-dependent manner (Figure 1B). Moreover, 24- and 48-hours treatment with high d-glucose increased PINK1 expression (Figure 1C), whereas 24 hours of l-glucose treatment did not affect PINK1 expression in SK-N-MC and SH-SY5Y cells or in mouse hippocampal neurons (Figure 1D-F). In addition, high glucose did not affect the expression of other mitophagy regulators, such as BNIP3 and NIX (Fig. S1). To assess the effect of high glucose on mitophagy, we determined the mitochondrial volume in SK-N-MC cells by using mitochondrial specific fluorescent dye Mitotracker™ and mitochondrial marker COX4. Immunofluorescence results showed that the fluorescence intensities of Mitotracker™ in SK-N-MC cells with high glucose treatment for 24 and 48 hours were reduced to 71.2% and 37.0% of the control level, respectively (Figure 1G). The fluorescence intensities of COX4 following 24 and 48 hours of high glucose decreased to 68.8% and 42.4%, respectively, of the control level (Figure 1H). In addition, high glucose treatment increased co-localization of COX4 with PINK1 or LC-3B (Figure 1I,J). We pretreated NH4Cl as an autophagy inhibitor prior to d-glucose incubation to confirm the effect of high glucose on mitophagy. Our result showed NH4Cl pretreatment recovered high glucose-reduced COX4 expression (Fig. S2). These results indicate that high glucose conditions trigger mitophagy, and mitophagy is dependent on the PINK1 pathway in neuronal cells.

3.2 | Protective role of PINK1 in mitochondrial dysfunction and apoptosis induced by high glucose

We investigated further the role of PINK1 in high glucose-induced mitophagy. As shown in the Figure 2A, immunofluorescence results revealed that a high glucose-reduced COX4 fluorescence intensity was recovered by PINK1 siRNA transfection with high glucose or vehicle. We observed that PINK1 siRNA transfection significantly decreased PINK1 mRNA expression levels of SK-N-MCs with high glucose or vehicle (Fig. S3). In addition, we investigated the role of high glucose-induced PINK1 in Mfn2 ubiquitination. Our result showed high glucose significantly increased Mfn2 ubiquitination and decreased Mfn2 expression, which were reversed by PINK1 silencing (Figure 2B). This finding presents a direct evidence for PINK1-induced mitophagy in SK-N-MCs under high glucose condition. To determine the role of high glucose in ROS accumulation, we measured intracellular and mitochondrial-specific ROS levels in SK-N-MC cells. As shown in the Figure 2C, intracellular ROS level increased to 183.7% of the control level after high glucose treatment and increased further to 276.6% of the control level after PINK1 siRNA transfection with high glucose treatment. Furthermore, ROS levels in SK-N-MCs with PINK1 siRNA transfection alone was increased to 170.2% of control. The flow cytometry results showed that silencing of PINK1 with high glucose or vehicle increased the number
of MitoSOX™-positive cells and decreased the number of TMRE-positive cells (Figure 2D,E). Taken together, these results indicate that high glucose-triggered mitophagy via PINK1 induction has an essential role in the suppression of mitochondrial ROS accumulation and the maintenance of mitochondrial quality control. Next, we investigated the role of high glucose-induced PINK1 in neuronal cell death. As shown in the Figure 3A, 25 and 50 mmol/L of d-glucose treatments stimulated cleaved caspase-3 and caspase-9 expressions. In support of those results, trypan blue exclusion assay results showed that 25 and 50 mmol/L d-glucose, not l-glucose, treatments reduced SK-N-MC cell viability (Figure 3B). To determine the role of high glucose-induced PINK1 in neuronal cell death, we transfected PINK1 siRNA into SK-N-MC cells. The western blot results showed that cleaved caspase-9 and -3 expressions in PINK1 siRNA-transfected
FIGURE 1  Effect of high glucose on PTEN-induced putative kinase 1 (PINK1)-induced mitophagy in neuronal cells. (A) SK-N-MCs were incubated with various concentrations of d-glucose (5–50 mmol/L) for 24 hour. The mRNA expressions of PINK1, Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and NIX were measured by quantitative real-time polymerase chain reaction. mRNA expression levels were normalized by ACTB mRNA expression level. Data are presented as a mean±SEM n=5. (B) The protein expressions of PINK1, cytochrome c oxidase subunit 4 (COX4) and β-actin were assessed by western blot. Relative optical density (ROD) of PINK1 and COX4 were normalized by β-actin. ROD data are presented as a mean±SEM n=4. (C) SK-N-MCs were incubated with various durations of 25 mmol/L of d-glucose (0–48 hour). The protein expressions of PINK1 and β-actin were detected by western blot. ROD data of PINK1 was normalized by β-actin. Quantitative ROD data are presented as a mean±SEM n=4. (D) SK-N-MCs, SH-SY5Ys and mouse primary hippocampal neurons were incubated with 25 mmol/L of d-glucose or 25 mmol/L of l-glucose for 24 hour. PINK1 and β-actin proteins were detected by western blot. ROD of PINK1 was normalized by β-actin. ROD data are presented as a mean±SEM n=4. (G) SK-N-MCs were incubated with 25 mmol/L of d-glucose (0–48 hour). Cells were immunostained with COX4-specific antibody and propidium iodide. The fluorescence intensities of COX4 are presented as a mean±SEM n=6 (magnification, ×800). All scale bars, 10 μm. (H) SK-N-MCs were incubated with 25 mmol/L of d-glucose for 24 hour. Cells were incubated with COX4, PINK1 and microtubule-associated proteins 1A/1B light chain 3B (LC-3B)-specific antibodies for 24 hour. The confocal images are representative (magnification, ×800). All scale bars, 10 μm. Each blot images are representative. *P<.05 vs control

3.3 | Effect of melatonin on PINK1-mediated mitophagy in neuronal cells

We investigated the effects of melatonin and high glucose on mitochondrial regulator expressions in SK-N-MC cells. In those cells, the mRNA expression of PINK1, but not BNIP3 or NIX, was significantly increased by melatonin treatment either with or without high glucose treatment (Figure 4A). In addition, western blot results showed that melatonin did not affect BNIP3 and NIX expressions (Fig. S4). Moreover, we observed that PINK1 protein expression induced by high glucose was further enhanced by melatonin pretreatment in SK-N-MC and SH-SY5Y cells and mouse hippocampal neurons (Figure 4B-D). Immunofluorescence results showed that COX4 fluorescence intensities in high glucose and after co-treatment of high glucose and melatonin were decreased to 75.5% and 31.6%, respectively, of the untreated level (Figure 4E). To confirm the effect of melatonin on mitochondrial ROS accumulation and mitochondrial membrane potential, we undertook MitoSOX™ and TMRE staining and measured the results by using flow cytometry. As shown in the Figure 4F,G, melatonin pretreatment decreased the number of MitoSOX™-positive cells and increased that of TMRE-positive cells. Collectively, our results indicate that melatonin facilitates PINK1-dependent mitophagy, which is associated with protection against mitochondrial impairment in neuronal cells under high glucose conditions.

3.4 | Involvement of MT2/Akt/NF-κB pathway in PINK1 induction by melatonin

Next, we investigated the mechanism involved in melatonin-induced regulation of PINK1 expression in neuronal cells. We observed the mRNA expressions of melatonin receptors, MTNR1A and MTNR1B, in SK-N-MC cells (Fig. S5). To confirm the effect of high glucose on melatonin receptor mRNA expressions, we incubated SK-N-MC cells with different concentrations of d-glucose and observed that 25 and 50 mmol/L of d-glucose-stimulated MTNR1B mRNA expression, which was reversed by pretreatment with ROS scavenger NAC (Figure 5A-C). Therefore, we focused on the role of MTNR1B in melatonin-induced PINK1 expression. As shown in the Figure 5D-F, PINK1 expression induced by melatonin (1 μmol/L) was abolished by pretreatment with the MT2 specific inhibitor 4-P-PDOT in SK-N-MC and SH-SY5Y cells and in mouse hippocampal neurons. Based upon these results, we suggest that high glucose induces MT2 expression via ROS signaling, and such expression is critical for melatonin’s action on PINK1 expression. We also checked 4-P-PDOT’s effect on PINK1 expression as a control in SK-M-MC, SH-SY5Y and mouse hippocampal neuron. And, our data showed that pretreatment of 4-P-PDOT did not affect high glucose-induced PINK1 expression in SK-M-MC, SH-SY5Y and mouse hippocampal neuron (Fig. S6A-C). We further investigated the signaling pathway regulating PINK1 expression induced by MT2 activation. The results showed that phosphorylation of Akt at the Thr389 and Ser473 residues increased to the greatest extent after 24 hours of d-glucose treatment (Figure 6A). High glucose phosphorylation of Akt, but not ERK or pan PKC, was further augmented by melatonin treatment (Figure 6B and Fig. S7). Our
FIGURE 2  Role of PTEN-induced putative kinase 1 (PINK1) in the reactive oxygen species (ROS) production and mitochondrial membrane potential in SK-N-MCs under high glucose. (A) PINK1 or nontargeting (NT) small interfering RNAs (siRNAs) were transfected to SK-N-MCs prior to 25 mmol/L of d-glucose treatment for 48 hour. Cells were immune-stained with cytochrome c oxidase subunit 4 (COX4)-specific antibody and propidium iodide. The fluorescence intensities of COX4 are reported as a mean±SEM n=6 (magnification, ×600). All scale bars, 50 μm. All confocal images are representative. (B) SK-N-MCs were transfected with PINK1 or NT siRNAs for 24 hour prior to d-glucose (25 mmol/L) for 24 hour. Mitofusin-2 (Mfn2) interacted proteins were immune-precipitated, and blotted with Ubiquitin and Mfn2 antibodies. Cell lysates as an input were blotted with Ubiquitin, Mfn2 and β-actin antibodies. All blot images are representative. Data are showed as a mean±SEM n=3. (C) SK-N-MCs were transfected with PINK1 or NT siRNAs for 24 hour prior to d-glucose (25 mmol/L) for 48 hour. Intracellular ROS level was analyzed by CM-H2DCF-DA (DCF-DA) staining. The fluorescence intensity of DCF-DA was detected by luminometer. Data are presented as a mean±SEM n=6. (D and E) The population of MitoSOX™ or tetramethylrhodamine ethyl ester-positive cells was measured by flowcytometer. Data are showed as a mean±SEM n=4. *P<.05 vs control with NT siRNA transfection, #P<.05 vs d-glucose treatment with NT siRNA transfection.
FIGURE 3  Role of high glucose-induced PTEN-induced putative kinase 1 (PINK1) expression in neuronal cell apoptosis. (A) SK-N-MCs were incubated with the various concentrations of d-glucose (0–50 mmol/L) for 48 hour. Cleaved caspase-9, cleaved caspase-3 and β-actin were detected by western blot. ROD data of cleaved caspase-9 and 3 were normalized by β-actin. Quantitative ROD data are presented as a mean±SEM n=3. (B) SK-N-MCs were incubated with 25 and 50 mmol/L of d-glucose or 50 mmol/L of l-glucose for 48 hour. Cell viability was measured by trypan blue exclusion cell viability assay. Data are presented as a mean±SEM n=6. (C) PINK1 or nontargeting small interfering RNAs were transfected to SK-N-MCs for 24 hour prior to 25 mmol/L of d-glucose treatment for 48 hour. Protein expressions of cleaved caspase-3 and β-actin were measured by western blot. ROD data of cleaved caspase-9 and 3 were normalized by β-actin. Quantitative ROD data are present as a mean±SEM n=3. (D) Cell viability was measured by trypan blue exclusion cell viability assay. Data are presented as a mean±SEM n=6. (E) Apoptotic cells were measured by annexin V/propidium iodide analysis assay. Data are reported as a mean±SEM n=4. Each blot images are representative. *P<.05 vs control, #P<.05 vs d-glucose treatment.
FIGURE 4  The effect of melatonin on PTEN-induced putative kinase 1 (PINK1) expression and mitochondrial function in neuronal cells under high glucose. (A) SK-N-MCs were incubated with d-glucose (25 mmol/L) and melatonin (1 μmol/L) for 24 hour. PINK1, Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and NIX mRNA expressions were measured by quantitative real-time polymerase chain reaction. The mRNA expression levels were normalized by ACTB mRNA expression. Data are presented as a mean±SEM n=5. (B-D) SK-N-MCs, SH-SY5Ys and mouse primary hippocampal neurons were incubated with 25 mmol/L of d-glucose and melatonin (1 μmol/L) for 24 hour. PINK1 and β-actin protein expressions were analyzed by western blot. ROD data of PINK1 was normalized by β-actin. Quantitative ROD data are presented as a mean±SEM n=4. *P<.05 vs control, #P<.05 vs d-glucose treatment.
data showed that high glucose treatment stimulated NF-κB phosphorylation at the Ser536 residue, with a further increase induced by melatonin treatment (Figure 6C). Melatonin treatment stimulated expressions of phosphorylated NF-κB and total NF-κB in the nucleus (Figure 6D). Furthermore, we also found that pretreatment of PI3K inhibitor LY294002 abolished enhancement of phosphorylation and nuclear translocation of NF-κB by melatonin (Figure 6C,D). To confirm the role of NF-κB activated by high glucose or melatonin in PINK1 expression, we pretreated NF-κB inhibitor SN-50 to melatonin-pretreated SK-N-MC cells. The results showed that enhanced mRNA and protein expressions of PINK1 by melatonin treatment were decreased by SN-50 (Figure 6E,F). Based upon our findings, we suggest that melatonin facilitates high glucose induction of Akt/NF-κB signaling via MT2 activation, thereby leading to PINK1 expression.
3.5 | Effect of PINK1 induced by melatonin on neuronal cell death

To determine the effect of melatonin-induced PINK1 expression on neuronal cell death under high glucose conditions, we investigated ROS regulation and neuronal cell apoptosis in PINK1 siRNA-transfected SK-N-MC cells with melatonin and high glucose treatments. As shown in the Figure 7A, the reduced intracellular ROS level in high glucose-incubated SK-N-MC cells induced by melatonin
FIGURE 7  Protective role of PTEN-induced putative kinase 1 (PINK1) expression enhanced by melatonin in high glucose-induced apoptosis in SK-N-MCs. (A) *PINK1* specific and nontargeting (NT) small interfering RNAs (siRNAs) were transfected to SK-N-MCs for 24 hour prior to d-glucose (25 mmol/L) and melatonin (1 μmol/L) for 48 hour. Intracellular reactive oxygen species level was measured by using CM-H_2DCF-DA (DCF-DA) staining. The fluorescence intensity of DCF-DA was detected by using luminometer. Data are presented as a mean±SEM n=6. (B) The protein expressions of cleaved caspase-9, cleaved caspase-3, PINK1 and β-actin were analyzed by western blot. The ROD data of cleaved caspase-9, cleaved caspase-3, and PINK1 were normalized by β-actin. Each blot images are representative. (C) Cell viability was analyzed by trypan blue exclusion cell viability assay. Data are presented as a mean±SEM n=6. (D) Apoptotic cells were measured by annexin V/propidium iodide analysis assay. Data are reported as a mean±SEM n=4. *P<.05 vs control with NT siRNA transfection, #P<.05 vs d-glucose treatment with NT siRNA transfection, @P<.05 vs co-treatment of d-glucose and melatonin with NT siRNA transfection. (E) Schematic model of for mechanism involved in the regulatory role of melatonin in high-glucose-induced PINK1 expression and neuronal cell survival.
treatment was reversed by PINK1 siRNA transfection. In addition, the increase in caspase-9 and -3 expressions induced by high glucose were significantly reversed by melatonin treatment, and reversed by PINK1 siRNA transfection in SK-N-MC cells (Figure 7B). Trypan blue exclusion assay results showed that cell viability of NT siRNA-transfected SK-N-MC cells given high glucose and melatonin was higher than that of NT siRNA-transfected SK-N-MC cells given high glucose alone. Moreover, this protective effect of melatonin against SK-N-MC cell apoptosis induced by high glucose was significantly reduced by PINK1 siRNA transfection (Figure 7C). Likewise, annexin V/PI staining flow cytometry results showed that melatonin significantly decreased the number of annexin V-positive cells in SK-N-MC cells treated with high glucose, reversed by PINK1 siRNA transfection (Figure 7D). Taken together, our results indicate that PINK1 has a crucial role in the anti-apoptotic effect of melatonin in neuronal cells.

4 | DISCUSSION

The results of this study highlight the mechanism of PINK1 regulation by melatonin in high glucose-induced neuronal cell death. Many investigators have reported that mitophagy is increased in various in vitro and in vivo DM models. In addition, previous research has presented evidence of relationships between PINK1 variation and disease. Although the majority of previous reports focused on PINK1 regulation by high glucose, a recent report showed that high glucose also increases BNIP3 expression, which contributes to cell survival in myocardial cells. In the present study, we have demonstrated that high glucose stimulates PINK1 expression, but not that of BNIP3 or NIX. These results may indicate that high glucose triggers PINK1-dependent mitophagy in neuronal cells. Interestingly, we found that PINK1 protein expression is increased in a dose-dependent manner although PINK1 mRNA expressions in 25 and 50 mmol/L d-glucose condition are similar. This inconsistency of our PINK1 results between PCR and western blot may be responsible for PINK1 stabilization increased by high glucose-induced ROS production and mitochondrial impairment. In addition, our results showed that high glucose stimulates co-localization of LC-3B or PINK1 with COX4 as well as stimulating expressions of LC-3B and PINK1. Those results are consistent with a recent report that platelet cells derived from DM patients have increased expression levels of LC-3 and PINK1, leading to protection against oxidative stress. It has also been demonstrated that PINK1/Parkin-mediated ubiquitination of substrates including Mfn, TOM, and VDAC trigger mitophagy through interaction with p62 or LC-3 even though PINK1 does not have an LC3-interacting region motif. Mitophagy which induces selective mitonchondrial clearance has been considered as a defense mechanism against impairment of mitochondria by cellular stress that can trigger apoptosis. Previous studies have shown that high glucose leads to neuronal cell apoptosis despite the induction of PINK1-dependent mitophagy that acts as a protective factor in neurodegeneration by preventing the apoptosis. These findings imply that PINK1-dependent mitophagy induced by high glucose is insufficient to inhibit the neuronal cell apoptosis. Furthermore, our results showed that PINK1 silencing enhanced cytotoxic effect of high glucose in neuronal cells via mitochondrial ROS production and dysfunction. Taken together, we suggest the partial protective role of PINK1 against neuronal cell apoptosis under high glucose condition and PINK1-dependent mitophagy as a key regulator of mitochondrial quality control and anti-apoptosis in neuronal cells under high glucose condition. Because of PINK1’s antioxidative effect, PINK1 induction has been suggested as a promising strategy for treatment of neuronal apoptosis in neurodegenerative diseases such as Parkinson’s disease. Although it has been reported that melatonin induces PINK1, the effect of melatonin on mitophagy regulators in human neuronal cells has not been described fully. In the present study, we confirmed a stimulatory effect of melatonin on PINK1, but not BNIP3 or NIX, in SK-N-MC cells. In addition, our results demonstrate that melatonin can be a significant inducer of PINK1-dependent mitophagy in neuronal cells. Melatonin’s antioxidative mechanism has been well documented, but several researchers have also indicated that melatonin has a conditional ROS stimulatory potential. This twofold effect of melatonin seems to vary according to glucose concentration, glucose treatment duration, and cell type. In the present study, our results showed melatonin treatment significantly suppressed mitochondrial ROS accumulation and mitochondrial membrane potential impairment in neuronal cell under high glucose conditions. Our results support those of Silvia Cristofanon et al. who reported that melatonin-activated NF-κB signaling pathway is responsible for a survival. In addition, we observed that high glucose stimulates MT2 expression, and that PINK1 induction by melatonin is dependent on the MT2 signaling pathway in SK-N-MC and SH-SY5Y cells and in mouse hippocampal neurons, a pathway that is critical for neuronal cell survival under high glucose conditions. This is the first report of MT2 being an initiating mediator of melatonin-induced PINK1 expression in neuronal cells. Similar to the neuroprotective effect of melatonin, a previous report showed that MT2 expression was increased in hippocampal region astrocytes exposed to ischemic injury, indicating that MT2 expression in neuronal cells may be associated with oxidative stress. We assumed that high glucose-induced ROS is associated
with the increase of MT\textsubscript{2} expression in our experimental condition. Actually, our data showed that NAC pretreatment reduced the increase of MT\textsubscript{2} receptor expression by high glucose. However, the further investigation is required to better understand the underlying mechanisms which are involved in MT\textsubscript{2} expression by high glucose-induced ROS in neuroblastoma cell lines. Taken together, current and previous results suggest that neuronal cells exposed to a high glucose condition have high sensitivity to melatonin, and that sensitivity is associated with MT\textsubscript{2}-specific physiological regulation of melatonin in DM patients. In addition, there have been several reports indicating distinct roles of melatonin receptors in the pathogenesis of neurological disorders. Indeed, previous studies have reported that increased MT\textsubscript{1} and MT\textsubscript{2} levels are associated with Alzheimer’s disease and ischemic neuronal injury, respectively, and that decreased MT\textsubscript{1} and MT\textsubscript{2} levels are observed in Parkinson’s disease.\textsuperscript{59–61} However, there are no reports describing a role of MT\textsubscript{1} in hyperglycemia-associated neuronal cell death. Further investigation will be required to elucidate the role of melatonin receptors in melatonin-based treatment of DM patients with neurologic complications.

Our findings suggest high glucose increased phosphorylations of Akt and NF-κB. It has been documented that increment of intracellular ROS activates PI3K/Akt signaling in the neuronal cells, which has a capacity for regulation of NF-κB activity.\textsuperscript{62,63} Those findings indicate that ROS is an upstream regulator of Akt/NF-κB pathway induced by high glucose treatment. Furthermore, our investigation revealed that Akt/NF-κB signaling activated by MT\textsubscript{2} is important for PINK1-mediated neuronal cell survival under high glucose conditions. The C-terminal motif of activated MT\textsubscript{2} directly binds to Akt, which leads to regulation of neuronal cell-related physiology including hypothalamus-liver communication, axonogenesis, and synaptic transmission.\textsuperscript{64,65} In addition, it has been reported that Akt phosphorylation by melatonin induces neuroprotective effect.\textsuperscript{66} Although several investigators have reported that MT\textsubscript{1} and heteromeric MT\textsubscript{1}/MT\textsubscript{2} receptors activate ERK1/2 and PKC pathways,\textsuperscript{67–69} we observed that melatonin treatment did not affect the phosphorylation levels of ERK1/2 and pan PKC (Fig. S4). In addition, our results showed the MT\textsubscript{2} stimulates PINK1 expression via augmentation of the high glucose-activated Akt/NF-κB pathway, which indicates that incorporation of melatonin-induced MT\textsubscript{2} signaling into high glucose signaling is required for PINK1 expression. A previous study identified the NF-κB binding site within the transcription start site of the human PINK1 gene promoter and suggested NF-κB as a significant regulator of PINK1 expression in neuronal cells.\textsuperscript{70} These findings suggest that activation of the Akt/NF-κB pathway by melatonin is crucial in the regulation of PINK1 expression. Meanwhile, another report described nuclear factor (erythroid-derived 2)-like 2 (NRF2) as a PINK1 inducer involved in neuronal cell survival under oxidative stress.\textsuperscript{71} This observation suggests the possibility that NRF2 may also contribute to PINK1 expression in neuronal cell protection against oxidative stress under high glucose conditions. For example, it has been reported that melatonin-activated MT\textsubscript{2} signaling has a regulatory role in the attenuation of memory impairment via a NRF2-associated antioxidative effect.\textsuperscript{72} Although the present study demonstrated that MT\textsubscript{2}-induced NF-κB is a key factor in PINK1 expression, additional research will be needed to determine the role of NRF2 in melatonin-induced PINK1 expression of neuronal cells.

In conclusion, we have demonstrated that melatonin enhances PINK1-dependent mitophagy via the MT\textsubscript{2}/Akt/NF-κB pathway, and such mitophagy is critical for high glucose-induced mitochondrial impairment and apoptosis in neuronal cells (Figure 7E). We propose the melatonin-induced PINK1 is a key factor in the regulation of ROS accumulation and anti-apoptosis in neuronal cells under high glucose conditions. To our knowledge, our investigation is the first to demonstrate a detailed mechanism controlling PINK1 expression by melatonin in neuronal cells under high glucose conditions. Identification of the pathways involved in controlling PINK1 by high glucose and melatonin will provide novel insights that will be useful in the development of therapeutic strategies for the treatment of hyperglycemia-associated neuronal cell death.

ACKNOWLEDGEMENTS

This research was supported by National R&D Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2013M3A9B4076541), and Next-Generation BioGreen 21 Program (No. PJ011141), Rural Development Administration, Republic of Korea.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

X. Onphachanh: Conception and design, Collection and/or assembly of data, Manuscript writing. H.J. Lee: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing. J.R. Lim: Conception and/or assembly of data, Y.H. Jung: Manuscript writing. J.S. Kim: Collection and/or assembly of data. C.W. Chae: Collection and/or assembly of data. S. Lee: Conception and design. A.A. Gabr: Collection and/or assembly of data. H.J. Han: Conception and design, Data analysis and interpretation, Manuscript writing.
REFERENCES

1. Moussavi S, Chatterji S, Verdes E, et al. Depression, chronic diseases, and decrements in health: results from the World Health Surveys. Lancet. 2007;370:851-858.

2. Fan F, Liu T, Wang X, et al. ClC-3 expression and its association with hyperglycemia induced HT22 hippocampal neuronal cell apoptosis. J Diabetes Res. 2016;2016:2984380.

3. Kumar P, Raman T, Swain MM, et al. Hyperglycemia-induced oxidative-nitrosative stress induces inflammation and neurodegeneration via augmented tuberous sclerosis complex-2 (TSC-2) activation in neuronal cells. Mol Neurobiol. 2017;54:238-254.

4. Den Heijer T, Vermeer SE, Van Dijk EJ, et al. Type 2 diabetes and atrophy of medial temporal lobe structures on brain MRI. Diabetologia. 2003;46:1604-1610.

5. Saravia FE, Beauquis J, Revisin Y, et al. Hippocampal neuropathology of diabetes mellitus is relieved by estrogen treatment. Cell Mol Neurobiol. 2006;26:943-957.

6. Vikram A, Tripathi DN, Kumar A, et al. Oxidative stress and inflammation in diabetic complications. Int J Endocrinol. 2014;2014:679754.

7. Sima AA. Encephalopathies: the emerging diabetic complications. Acta Diabetol. 2010;47:279-293.

8. Tang Y, Liu J, Long J. Phosphatase and tensin homolog-induced apoptosis. Cell Res. 2013;17:719-730.

9. Gandhi S, Wood-Kaczmar A, Yao Z, et al. PINK1-associated susceptibility gene Clec16a regulates mitophagy. Cell Metab. 2012;61:96-107.

10. Frank M, Duvezin-Caubet S, Koob S, et al. Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner. Biochim Biophys Acta. 2012;1823:2297-2310.

11. Liu L, Sakakibara K, Chen Q, et al. Receptor-mediated mitophagy in yeast and mammalian systems. Cell Res. 2014;24:787-795.

12. Melser S, Chatelain EH, Lavie J, et al. Rheb regulates mitophagy induced by mitochondrial energetic status. Cell Metab. 2013;17:719-730.

13. Gandhi S, Wood-Kaczmar A, Yao Z, et al. PINK1-associated Parkinson’s disease is caused by neuronal vulnerability to calcium-induced cell death. Mol Cell. 2009;33:627-638.

14. Bellot G, Garcia-Medina R, Gounon P, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol. 2009;29:2570-2581.

15. Soleimanpour SA, Gupta A, Bakay M, et al. The diabetes susceptibility gene Clec16a regulates mitophagy. Cell. 2014;157:1577-1590.

16. Hoshino A, Ariyoshi M, Okawa Y, et al. Inhibition of p53 preserves Parkin-mediated mitophagy and pancreatic β-cell function in diabetes. Proc Natl Acad Sci USA. 2014;111:3116-3121.

17. Lee SH, Du J, Sthitham J, et al. Inducing mitophagy in diabetic platelets protects against severe oxidative stress. EMBO Mol Med. 2016;8:779-795.

18. Lo MC, Lu CI, Chen MH, et al. Glycoxidative stress-induced mitophagy modulates mitochondrial fates. Ann N Y Acad Sci. 2010;1201:1-7.

19. Manchester LC, Coto-Montes A, Boga JA, et al. Melatonin: an ancient molecule that makes oxygen metabolically tolerable. J Pineal Res. 2015;59:403-419.

20. Reiter RJ, Mayo JC, Tan DX, et al. Melatonin as an antioxidant: under promises but over delivers. J Pineal Res. 2016;61:253-278.

21. Jockers R, Maurice P, Boutin JA, et al. Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? Br J Pharmacol. 2008;154:1182-1195.

22. Staiger H, Machicau F, Schafer SA, et al. Polymorphisms within the novel type 2 diabetes risk locus MTNR1B determine β-cell function. PLoS ONE. 2008;3:e3962.

23. Lysenkov V, Nagorny CL, Erdos MR, et al. Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. Nat Genet. 2009;41:82-88.

24. Bonnefond A, Froguel P. The case for too little melatonin signaling in increased diabetes risk. Diabetologia. 2017;60:823-825.

25. Costes S, Boss M, Thomas AP, et al. Activation of melatonin signaling promotes β-cell survival and function. Mol Endocrinol. 2015;29:682-692.

26. Muennan CJ, Schernhammer ES, Rimm EB, et al. Melatonin secretion and the incidence of type 2 diabetes. JAMA. 2013;309:1388-1396.

27. Tuomi T, Nagorny CL, Singh P, et al. Increased melatonin signaling is a risk factor for type 2 diabetes. Cell Metab. 2016;23:1067-1077.

28. Mattson MP. Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol. 2000;1:120-129.

29. Chen ST, Chuang JI. The antioxidant melatonin reduces cortical neuronal death after intrastratal injection of kainate in the rat. Exp Brain Res. 1999;124:241-247.

30. Antolin I, Mayo JC, Sainz RM, et al. Protective effect of melatonin in a chronic experimental model of Parkinson’s disease. Brain Res. 2002;943:163-173.

31. Coto-Montes A, Boga JA, Rosales-Corral S, et al. Role of melatonin in the regulation of autophagy and mitophagy: a review. Mol Endocrinol. 2012;36:12-23.

32. Kang JW, Hong JM, Lee SM. Melatonin enhances mitophagy and mitochondrial biogenesis in rats with carbon tetrachloride-induced liver fibrosis. J Pineal Res. 2016;60:383-393.

33. Wang P, Sun X, Wang N, et al. Melatonin enhances the occurrence of autophagy induced by oxidative stress in Arabidopsis seedlings. J Pineal Res. 2015;58:479-489.

34. Diaz-Casado ME, Lima E, Garcia JA, et al. Melatonin rescues zebrafish embryos from the parkinsonian phenotype restoring the parkin/PINK1/DJ-1/MUL1 network. J Pineal Res. 2016;61:96-107.

35. Lee HJ, Ryu JM, Jung YH, et al. High glucose upregulates BACE1-mediated Aβ production through ROS-dependent HIF-1α and LXRα/ABCA1-regulated lipid raft reorganization in SK-N-MC cells.Sci Rep. 2016;6:36746.

36. Li Y, Xu S, Zhang Q, et al. Cytotoxicity study on SHSY5Y cells cultured at high glucose levels and treated with bupivacaine. Mol Med Rep. 2014;9:515-520.

37. Wang X, Yu S, Wang CY, et al. Advanced glycation end products induce oxidative stress and mitochondrial dysfunction in SH-SYSY cells. In Vitro Cell Dev Biol Anim. 2015;51:204-209.

38. Suzuki M, Sasabe J, Furuya S, et al. Type 1 diabetes mellitus in mice increases hippocampal D-serine in the acute phase after streptozotocin injection. Brain Res. 2012;1466:167-176.

39. Stoving H, Harmsen CG, Wisloff T, et al. A competing risk approach for the European Heart SCORE model based on cause-specific and all-cause mortality. Eur J Prev Cardiol. 2013;20:827-836.
40. Moruno F, Perez-Jimenez E, Knecht E. Regulation of autophagy by glucose in mammalian cells. Cells. 2012;1:372-395.
41. Scheele C, Nielsen AR, Walden TB, et al. Altered regulation of the PINK1 locus: a link between type 2 diabetes and neurodegeneration? FASEB J. 2007;21:3653-3665.
42. Zhou W, Yang J, Zhang DI, et al. Role of Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 in myocardial cells in diabetes. Exp Ther Med. 2015;10:67-73.
43. Narendra DP, Jin SM, Tanaka A, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010;8:e1000298.
44. Saito T, Sadoshima J. Molecular mechanisms of mitochondrial autophagy/mitophagy in the heart. Circ Res. 2015;116:1477-1490.
45. Grenier K, Mclelland GL, Fon EA. Parkin- and PINK1-dependent mitophagy in neurons: will the real pathway please stand up? Front Neurol. 2013;4:100.
46. Narendra D, Kane LA, Hauser DN, et al. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy. 2010;6:1090-1106.
47. Okatsu K, Saisho K, Shimanuki M, et al. p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. Genes Cells. 2010;15:887-900.
48. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res. 2012;111:1208-1221.
49. Meng X, Wang X, Tian X, et al. Protection of neurons from high glucose-induced injury by deletion of MAD2B. J Cell Mol Med. 2014;18:844-851.
50. Lee S, Zhang C, Liu X. Role of glucose metabolism and ATP in maintaining PINK1 levels during Parkin-mediated mitochondrial damage responses. J Biol Chem. 2015;290:904-917.
51. Moisioli N, Fedele V, Edwards J, et al. Loss of PINK1 enhances neurodegeneration in a mouse model of Parkinson’s disease triggered by mitochondrial stress. Neuropharmacology. 2014;77:350-357.
52. Anichtchik O, Diekmann H, Fleming A, et al. Loss of PINK1 function affects development and results in neurodegeneration in zebrafish. J Neurosci. 2008;28:8199-8207.
53. Pidgeon JW, Olzmann JA, Chiu LS, et al. PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. PLoS Biol. 2007;5:e172.
54. Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004;304:1158-1160.
55. Petit A, Kawarai T, Paitel E, et al. Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. J Biol Chem. 2005;280:34025-34032.
56. Wood-Kaczmar A, Gandhi S, Yao Z, et al. PINK1 is necessary for long term survival and mitochondrial function in human dopaminergic neurons. PLoS ONE. 2008;3:e2455.
57. Zhang HM, Zhang Y. Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. J Pineal Res. 2014;57:131-146.
58. Cristofanon S, Uguccioni F, Cerella C, et al. Intracellular prooxidant activity of melatonin induces a survival pathway involving NF-κB activation. Ann N Y Acad Sci. 2009;1171:472-478.
59. Lee CH, Yoo KY, Choi JH, et al. Melatonin’s protective action against ischemic neuronal damage is associated with up-regulation of the MT2 melatonin receptor. J Neurosci Res. 2010;88:2630-2640.
60. Savaskan E, Olivieri G, Brydon L, et al. Cerebrovascular melatonin MT1-receptor alterations in patients with Alzheimer’s disease. Neurosci Lett. 2001;308:9-12.
61. Adi N, Mash DC, Ali Y, et al. Melatonin MT1 and MT2 receptor expression in Parkinson’s disease. Med Sci Monit. 2010;16:BR61-BR67.
62. Sadiki M, Lentz SI, Feldman EL, Hydrogen peroxide-induced Akt phosphorylation regulates Bax activation. Biochimie. 2009;91:577-585.
63. Bai D, Ueno L, Vogt PK. Akt-mediated regulation of NFκB and the essentialness of NFκB for the oncogenicity of PI3K and Akt. Int J Cancer. 2009;125:2863-2870.
64. Anhe GF, Caperuto LC, Pereira-Da-Silva M, et al. In vivo activation of insulin receptor tyrosine kinase by melatonin in the rat hypothalamus. J Neurochem. 2004;90:559-566.
65. Liu D, Wei N, Man HY, et al. The MT2 receptor stimulates axonogenesis and enhances synaptic transmission by activating Akt signaling. Cell Death Differ. 2015;22:583-596.
66. Kong PJ, Byun JS, Lim SY, et al. Melatonin induces Akt phosphorylation through melatonin receptor- and PI3K-dependent pathways in primary astrocytes. Korean J Physiol Pharmacol. 2008;12:37-41.
67. Luchetti F, Canonico B, Betti M, et al. Melatonin signaling and cell protection function. FASEB J. 2010;24:3603-3624.
68. Radio NM, Doctor JS, Witt-Enderby PA. Melatonin enhances alkaline phosphatase activity in differentiating human adult mesenchymal stem cells grown in osteogenic medium via MT2 melatonin receptors and the MEK/ERK (1/2) signaling cascade. J Pineal Res. 2006;40:332-342.
69. Baba K, Benleulmi-Chaachoua A, Journe AS, et al. Heteromeric MT1/MT2 melatonin receptors modulate photoreceptor function. Sci Signal. 2013;6:ra89.
70. Duan X, Tong J, Xu Q, et al. Upregulation of human PINK1 gene expression by NFκB signalling. Mol Brain. 2014;7:57.
71. Murata H, Takamatsu H, Liu S, et al. NRF2 regulates PINK1 expression under oxidative stress conditions. PLoS ONE. 2015;10:e0142438.
72. Shin EJ, Chung YH, Le HL, et al. Melatonin attenuates memory impairment induced by Klotho gene deficiency via interactive signaling between MT2 receptor, ERK, and Nrf2-related antioxidant potential. Int J Neuropsychopharmacol. 2014;18.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.