Melatonin prevents cytosolic calcium overload, mitochondrial damage and cell death due to toxically high doses of dexamethasone-induced oxidative stress in human neuroblastoma SH-SY5Y cells

Wilasinee Suwanjang[a,b], Andrey Y. Abramov[c], Komgrid Charngkaew[d], Piyarat Govitrapong[b,e], Banthit Chetsawang[b]

[a]Center for Innovation Development and Technology Transfer, Faculty of Medical Technology, and [b]Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Nakhonpathom, 73170 Thailand

[c]Department of Molecular Neuroscience, Institute of Neurology, UCL, Queen Square, London WC1N 3BG, UK

[d]Department of Pathology, Faculty of Medicine Siriraj Hospital, Bangkok 10700, Thailand

[e]Center for Neuroscience and Department for Pharmacology, Faculty of Science, Mahidol University, Bangkok, 10400 Thailand

Corresponding Author:

Banthit Chetsawang, Ph.D.

Research Center for Neuroscience,

Institute of Molecular Biosciences, Mahidol University,

Salaya, Nakhonpathom 73170, Thailand

Tel. +66-2-4419003-7

Fax. +66-2-4411013

Email: banthit.che@mahidol.ac.th

Key words: melatonin; mitochondrial damage; cytosolic calcium overload; oxidative stress; dexamethasone; neuroprotection

Abstract

Stressor exposure activates the hypothalamic-pituitary-adrenal (HPA) axis and causes elevations in the levels of glucocorticoids (GC) from the adrenal glands. Increasing evidence has demonstrated that prolonged exposure to high GC levels can lead to oxidative stress, mitochondrial dysfunction and apoptosis in a number of cell types. However, melatonin, via its antioxidant activity, exhibits a neuroprotective effect against oxidative stress-induced cell death. Therefore, in the present study, we explored the protective effect of melatonin in GC-induced toxicity in human neuroblastoma SH-SY5Y cells. Cellular treatment with the toxically high doses of the synthetic GC receptor agonist, dexamethasone (DEX) elicited marked decreases in the levels of glutathione and increases in ROS production, lipid peroxidation and cell death. DEX toxicity also induced increases in the levels of cytosolic calcium and mitochondrial fusion proteins. Mitochondrial damage was observed in large proportions of the DEX-treated cells. Pretreatment of the cells with melatonin substantially prevented the DEX-induced toxicity. These results suggest that melatonin might exert protective effects against oxidative stress, mitochondrial damage and cytosolic calcium overload in DEX-induced neurotoxicity.

1. Introduction

Stressor exposure leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis and induces the secretion of the glucocorticoid stress hormones (GCs; cortisol in human, corticosterone (CORT) in rats), which exert widespread effects on many systems, including the brain [1]. In humans, stress-induced toxicity has been observed in many neurodegenerative diseases, including ischemic infarction [2]. Clinical investigations suggest that patients with Alzheimer's disease (AD) and Parkinson's disease (PD) exhibit higher total plasma cortisol concentrations [3]. Higher GCs levels lead to increases in reactive oxygen species (ROS) production that directly cause mitochondria dysfunction, decrease cellular energy yield, elevate cytosolic Ca^{2+} concentrations and alter mitochondrial permeability, which leads to apoptosis in neuron cells [4].

Many studies have provided compelling evidence that mitochondrial dysfunction plays a causative role in neurodegeneration. It has been demonstrated that mitochondrial dysfunctions are caused by imbalances in the mitochondrial dynamics of fission and fusion. [5]. Mitochondrial fission events are required in dividing cells and are also important during differentiation and in response to new energy demands and toxin exposures. Mitochondrial fusion (the process opposing fission) plays important roles in development and cell biology and also plays a protective role in apoptosis [6].

Melatonin is substance that, in the brain, is primarily synthesized and secreted by the pineal gland. In addition to its major role in circadian rhythm regulation, melatonin has been found to possess free radical scavenging properties and to effective in protecting cells from oxidative damage [7]. Previous studies have shown that melatonin can prevent oxidative stress-induced neuronal damage in dexamethasone (DEX)-treated SH-SY5Y cells [8]. Until recently, the exactly mechanisms by which stress and the GC stress hormone contribute to the

activation of cell death processes have remained unclear. Therefore, in the present study, we proposed a mechanism of the regulation of neuronal cell death by the synthetic GC receptor agonist DEX that is mediated via stress-induced cytosolic calcium overload and mitochondrial damage. Mitochondrial damage is dependent on imbalances in the dynamic mitochondrial processes of fission and fusion processes. Additionally, we also propose that melatonin has protective effects against GC-induced neurotoxicity.

2. Material and methods

2.1. Chemicals and Reagents

Melatonin was obtained from Sigma Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-actin was purchased from Chemicon International (Temecula, CA, USA). Mouse monoclonal anti-Fis1, anti-Drp1, anti-Mfn1 and anti-Opa1 were purchased from Santa Cruz Biotechnology, Inc. All other chemicals used in this study were analytical grade and obtained essentially either from Sigma Aldrich or Lab-Scan Analytical Science (Dublin, Ireland).

2.2. Cell cultures

Human neuroblastoma (SH-SY5Y) cells were maintained in 75-cm^2 flasks with minimum essential medium (MEM)-F12, supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2 and 95% air. For the experiments, the cells were seeded in 96-well and 6-well plates and grown to 70-80% confluence. Before the initiation of treatment, the media were replaced with MEM containing 1% (v/v) FBS.

2.3. Reactive oxygen species (ROS) assay

Cytosolic ROS production was measured by dihydroethidium (HEt) fluorescence. Fluorescence measurements were obtained with an epifluorescence inverted microscope

equipped with a 20x fluorite objective. HEt was monitored in single cells using excitation light provided by a Xenon arc lamp. All imaging data were collected and analyzed using the Kinetic Imaging (Wirral, UK) software. All presented data were obtained from at least five coverslips and three different cell preparations.

2.4. Glutathione measurements

The cells were incubated with 50 µM monochlorobimane (MCB) in HBSS at room temperature for 40 min or until a steady state had been reached before images were acquired for quantitation. The images of the fluorescence of the MCB_GSH adduct were acquired using either a cooled CCD imaging system with excitation at 380 nm and emission at > 400 nm or a Zeiss UV-vis 510 CLSM with excitation at 351 nm and emission at 435-485 nm.

2.5. Lipid peroxidation assay

The cells were loaded with 5 µM C11-BODIPY (581/591) for 30 min. The C11-BODIPY dye incorporates into the cytoplasmic membrane and responds to eventual lipid peroxidation by altering its fluorescence properties [17]. C11-BODIPY (581/591) was excited using the 488 nm and 543 nm laser lines, and fluorescence was measured using a bandpass filter of 505-550 nm and a 560-nm longpass filter. All data presented were obtained from four different cell preparations.

2.6. Cytosolic calcium ($[Ca^{2+}]_c$) determination

SH-SY5Y cells were loaded with 5 µM fluo-4 AM and 0.005% pluronic in a HBSS for 30 min at room temperature, $[Ca^{2+}]_c$ was then measured. The fluo-4 signal was excited at 490 nm and measured above 515 nm. Cytosolic calcium was measured under an FV10i confocal microscope (Olympus, Bio Imaging Center, Thailand).

2.7. Western immunoblotting

The cells were grown to the subconfluent stage in six-well plates. The cells were lysed by the addition of lysis buffer and scraped off the plate. The cells were sonicated for 10 s and centrifuged for 15 min at 12,000 g. The supernatants were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer (5% non-fat dry milk in TBST), and then incubated in the primary antibodies at 4ºC overnight. The membranes were incubated in HRP-conjugated secondary antibody for 1.30 hr. The blots were developed with Chemiluminescent ECL Plus Western Blotting detection reagents.

2.8. Cytotoxicity assay

The cells were exposed to 5 µM propidium iodide (PI), which exhibits a red fluorescence. PI is excluded from viable cells and thus stains cells that have lost their membrane integrity. Some cells were exposed to 5 µM Hoechst 33342, which stains chromatin blue, to count the total numbers of cells. This process allowed us to express of the number of dead (red-stained) cells as a fraction of the total number of nuclei that were counted. Total numbers of 100-300 SH-SY5Y cells were counted in 4-5 fields of each coverslip. Each experiment was repeated four or more times using separate cultures.

2.9. Transmission electron microscopy (TEM)

The SH-SY5Y cells were grown in 75-cm^2 culture flask. The cells were fixed with ice-cold 4% phosphate-buffered glutaraldehyde for 30-60 min. The fixed cell cultures were then rinsed three times for 10 min each with 0.1 M PBS. The cells were post-fixed with 2% phosphate-buffered osmium tetroxide for 30 min at room temperature and then washed two times for 10 min each with distilled water at room temperature. The cells were stained with 2% aqueous saturated uranyl acetate for 15 min at room temperature. The cells were then

dehydrated in graded ethanol baths and embedded in resin. Ultrathin (95-100 nm) sections were post-stained with uranyl acetate and evaluated under TEM.

2.10. Statistical analyses

The data are expressed as the mean ± the S.E.M. Significance was assessed with one-way analyses of variance (ANOVAs) followed by Tukey-Kramer tests using the scientific statistical software SPSS version 16. Probability (*P*) values below 0.05 were considered to indicate statistical significance.

3. Results

The effects of DEX treatment on ROS production were investigated in the SH-SY5Y cultured cells. The cells that were treated with 1 and 2 μM DEX for 90 min exhibited significant elevations of ROS production compared with the (0 μM DEX) control value (Fig. 1A). The NADPH oxidase inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 20 μM) and diphenyleneiodonium (DPI, 0.5 μM), lipoxygenase inhibitor NDGA (10 μM) and xanthine oxidase inhibitor oxypurinol (10 μM) were applied to the cultured cells prior to treatment with DEX. AEBSF and DPI significantly diminished the DEX-induced increases in ROS production compared with the DEX-treated cells, respectively (Fig. 1B). The results demonstrated that exposure to 1 μM DEX for 24 hr significantly decreased the amount of GSH compared with the control-untreated cells (Fig. 1C). The addition of 1 μM DEX significantly increased the rate of lipid peroxidation formation compared with the level observed in the control-untreated SH-SY5Y cells (Fig. 1D). The viable cells were quantified with using propidium iodide (PI) staining (Fig. 1F). Treatment with 1.0 μM DEX for 24 hr significantly increased neuronal cell death compared with the control-untreated cells. Pretreatment with 0.25 mM melatonin significantly

decreased neuronal cell death compared with the DEX-treated cells without melatonin (Fig. 1E).

The applications of 1 μM DEX for 18 and 24 hr significantly increased $[Ca^{2+}]_c$ compared with the levels observed in the DEX-treated cells at 0 hr (Fig. 2A). The cytosolic green fluorescence calcium staining signals were recorded in SH-SY5Y cells (Fig. 2B). Thus, treatment with 1 μM DEX for 24 hr significantly increased the cytosolic calcium level compared with the control value. Pretreatment with 0.25 mM melatonin was diminished this DEX-induced increase in cytosolic calcium. Melatonin alone had no effect on cytosolic calcium compared with the control-untreated cells (Fig. 2C).

The effects of DEX-induced alterations of mitochondrial fission (Drp1 and Fis1) and mitochondrial fusion (Opa1 and Mfn1) proteins were determined in the SH-SY5Y cells using Western blot analyses. Treatment with 1.0 μM DEX for 24 hr significantly decreased the Drp1 expression compared with the control value. Additionally, treatments with 1.0 μM DEX for 1, 2, 12, 18 and 24 hr significantly decreased the amounts of Fis1 expression compared with the control value. Treatment with 1.0 μM DEX for 12 and 18 hr significantly increased the amount of Opa1 and Mfn1 compared with the control value, respectively (Fig. 3A). SH-SY5Y cells were exposed to 1 μM DEX for the indicated times with or without pre-treatment with 0.25 mM melatonin for 1 hr (Fig. 3B-C). However, pretreatment of the SH-SY5Y cells with 0.25 mM melatonin for 1 hr prior to treatment with 1 μM DEX significantly increased the amounts of Drp1 and Fis1 (Fig. 3B) and significantly decreased the amounts of Opa1 and Mfn1(Fig. 3C) compared with the DEX-treated cells, respectively. Melatonin at 0.25 mM alone had no effect on the amounts of either the mitochondrial fission (Fig. 3B) or fusion (Fig. 3C) proteins in the SH-SY5Y cells compared to the control.

The protective effects of melatonin on DEX-induced alterations in mitochondrial morphology were examined using transmission electron microscopy (TEM). Control-untreated cells exhibited both rod and elongate mitochondrial shapes. The matrix was fairly dark, and the cristae were regularly distributed (Fig. 4B arrows). The DEX-treated group exhibited electron-dense round, abnormal mitochondrial morphologies (Fig. 4D arrows) and a greater proportion elongated mitochondria shape (Fig. 4C arrows). Mitochondrial swelling, few remaining cristae and discontinuous outer membranes were also observed in the DEX-treated cells (Fig. 4D arrowheads). Pretreatment with melatonin resulted in normal cristae structures and normal mitochondrial morphologies (Fig. 4E arrows).

4. Discussion

Long-term treatment with DEX has been proven to induce ROS production [9], and oxidative stress is recognized as a potent inducer of decreases in cell function and eventual cell death [10]. In the present study, toxically high doses of DEX were observed to induce increases in ROS formation in the dopaminergic SH-SY5Y cell line. Excessive production of ROS is the earliest sign of disturbed homeostasis that leads to neuronal cell death [11]. Two lines of evidence support the role of oxidative damage in DEX-induced toxicity in dopaminergic cells. First, it has been reported that DEX-treated murine neural stem cells exhibit decreased expressions of the antioxidant enzymes catalase and superoxide dismutase 1 (SOD1), which results in the failures of antioxidant functions [12]. Second, DEX has been documented to increase monoamine oxidase (MAO) activity in SH-SY5Y cells [13] and in the rat substantia nigra [14], which leads to increases in intracellular dopamine oxidative deamination. Increases in ROS formation by DEX could, at least in part, be due to increases in dopamine oxidation by MAO [14]. It seems that DEX toxicity induces an imbalance that involves increased ROS production and reduced ROS scavenging. Taken together, our findings indicated that DEX-induced increases in ROS formation might be a part of a

mitochondria-dependent process as indicated by the ability of the NADPH-oxidase inhibitor (AEBSF and DPI) to reduce ROS formation. Excessive amounts of ROS induced oxidative damage to polyunsaturated fatty acids, which was observed as an increase in lipid peroxidation during DEX-induced toxicity. Supporting these hypotheses, reductions in antioxidant enzymes and glutathione levels or the induction of lipid peroxidation were demonstrated in the SH-SY5Y cultured cells. Similarly, considerable evidence suggests that glutathione depletion plays a major role in the progression toward cell death. Further induction of lipid peroxidation is produced by the breakdown of polyunsaturated fatty acids [15].

The protective effects of melatonin and its metabolites have been demonstrated in a variety of oxidative stress-associated neuropathologies. Currently, it well documented that melatonin prevents the neurotoxin-induced deaths of hippocampal [16] and neuroblastoma cells [8]. Increasing amount of evidence support the neuroprotective effect of melatonin against calcium-dependent cell death cascades [17]. In the present study, we found that melatonin reversed the toxic insult of DEX in SH-SY5Y cells by decreasing cytotoxic calcium overload and cell death rates in dopaminergic SH-SY5Y cells. These results revealed the protective role of melatonin against DEX-induced oxidative stress and calcium-dependent death processes in SH-SY5Y cells. The present data corroborate those of previous studies in which melatonin was found to regulate intracellular processes, such as G-proteins, and the activities of second messengers, such as cAMP, IP3 and Ca^{2+} [18]. The Ca^{2+} signaling system modulates receptors and voltage-dependent calcium channels, pumps, exchangers and binding proteins [19]. Melatonin can prevent ischemic injury-induced reductions in the levels of the calcium-buffering proteins parvalbumin and hippocalcin in the cerebral cortical tissue of rats [17]. Recently, Espino et al. demonstrated the protective effect of melatonin supplementation against ER-stress-induced apoptosis driven by calcium signaling in human

leukocytes. It has been proposed that melatonin is able to delay calcium overload-induced apoptosis due to its antioxidant properties [20]. This postulated role of melatonin is consistent with evidence that has shown that ROS, such as H_2O_2, increase cytosolic calcium release from intracellular pools, which leads to apoptotic states [21].

More importantly, we demonstrated that melatonin is able to diminish decreases in mitochondrial fission protein levels (Drp1 and Fis1) and increase mitochondrial fusion protein levels (Opa1 and Mfn1) in DEX-treated cells. Melatonin was able to prevent mitochondrial damage and preserve the mitochondrial membrane potential and energy production during cell stress [22]. We also examined the morphological alterations of the mitochondria with transmission electron microscopy. The DEX-treated cells exhibited numerous electron-dense, round, abnormal mitochondrial structures. Our data suggest that DEX play a critical role in activating ROS that are associated with mitochondrial fission-fusion imbalances and cause progression to cell death. Excessive mitochondrial fusion seems to be important for DEX-induced neurotoxicity. Recent evidence has emphasized that excessive mitochondrial fusion and fission-fusion imbalances might lead to mitochondrial dysfunction [23]. It has been demonstrated that excessive fused mitochondria accumulate oxidative damage and further transform into large spheres. These large round mitochondria are associated with impaired function [24]. Our observations suggest that melatonin might exert its neuroprotective effects not only by inhibiting ROS generation but also by maintaining mitochondrial fission-fusion and cytosolic calcium homeostasis, which might reduce cell degeneration following DEX exposure.

Acknowledgements

This work was supported by a research grant from Mahidol University and the Thailand Research Fund (TRF) under the TRF-Basic Research Grant (BRG5780003) to BC,

TRF-Distinguish Research Professor (DPG5780001) to PG and TRF grant to the Institute of Molecular Biosciences (IRG5780009).

References

[1] B. McEwen, Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators, Eur. J. Pharmacol. 583 (2008) 174-185.

[2] K. Niizuma, H. Endo, P. Chan, Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival, J. Neuochem. 109 (2009) 133-138.

[3] A. Hartmann, J. Veldhuis, M. Deuschle, H. Standhardt, I. Heuser, Twenty-four hour cortisol release profiles in patients with Alzheimer's and Parkinson's disease compared to normal controls: ultradian secretory pulsatility and diurnal variation, Neurobiol. Aging 18 (1997) 285-289.

[4] D. Filipovic, J. Zlatkovic, D. Inta, I. Bjelobaba, M. Stojiljkovic, P. Gass, Chronic isolation stress predisposes frontal cortex but not the hippocampus to the potentially deterimental release of cytochrome c from mitochondria and the activation of caspase-3, J. Neurosci. Res. 89 (2011) 1461-1470.

[5] H. Chen, D. Chan, Mitochondrial dynamics-fusion, fission, movement and mitophagy-in neurodegenerative diseases, Hum. Mol. Denet. 18 (2009) R169-176.

[6] C. Frezza, S. Cipolat, B.d. Martins, M. Micaroni, G.V. Beznoussenko, T. Rudka, D. Bartoli, R.S. Polishuck, N.N. Danial, OPA1 controls apoptosis cristae remodeling independently from mitochondrial fusion, Cell 126 (2006) 177-189.

[7] F. Huang, H. Ning, Q. Xin, Y. Huang, H. Wang, Z. Zhang, D.X. Xu, G. Ichihara, D.Q. Ye, Melatonin pretreatment attenuates 2-bromopropane-induced testicular toxicity in rats, Toxicology 256 (2009) 75-82.

[8] W. Suwanjang, A. Abramov, P. Govitrapong, B. Chetsawang, Melatonin attenuates dexamethasone toxicity-induced oxidative stress, calpain and caspase activation in human neuroblastoma SH-SY5Y cells, J. Steroid Biochem. Mol. Biol. 138 (2013) 116-122.

[9] K. Asayama, H. Hayashibe, K. Dobashi, N. Uchida, K. Kato, Effect of dexamethasone o antioxidant enzymes in fetal rat lungs and kidneys, Biol Neonate. 62 (1992) 136-144.

[10] K. Loh, S. Huang, R.D. Silva, B. Tan, Y. Zhu, Oxidative stress: apoptosis in neuronal injury, Curr. Alzheimer Res. 3 (2006) 327-337.

[11] A. Valencia, J. Moran, Reactive oxygen species induce different cell death mechanisms in cultured neurons, Free Radic. Biol. Med. 36 (2004) 1112-1125.

[12] H.A. Mutsaers, R. Tofighi R, Dexamethasone enhances oxidative stress-induced cell death in murine neural stem cells, Neurotox. Res. 22 (2012) 127-137

[13] S. Johnson, S. Tazik, D. Lu, C. Johnson, M. Youdim, J. Wang, G. Rajkowska, X. Ou, The new inhibitor of monoamine oxidase, M30 has a neuroprotective effect against dexamethasone-induced brain cell apoptosis, Front. Neurosci. 4 (2010) 1-6.

[14] S. Arguelles, A. Herrera, E. Carreno-Muller, R.d. Pablos, R. Villaran, A. Espinosa, A. Machado, J. Cado, Degeneration of dopaminergic neurons induced by thrombin injection in the substantia nigra of the rat is enhanced by dexamethasone: Role of monoamine oxidase enzyme, Neurotoxicology 31 (2010) 55-66.

[15] A. Abramov, L. Canevari, M. Duchen, Calcium signals induced by amyloid B peptide and their consequences in neurons and astrocytes in culture, Biochim. Biophys. Acta. 1742 (2004) 81-87.

[16] K. Permpoonputtana, S. Mukda, P. Govitrapong, Effect of melatonin on D-amphetamine-induced neuroglial alterations in postnatal rat hippocampus and prefrontal cortex, Neurosci. Lett. 524 (2012) 1-4.

[17] P. Koh, Melatonin regilate the calcium-buffering proteins, parvalbumin and hippocalcin, in ischemic brain injury, J. Pineal Res. 53 (2012) 358-365.

[18] J.d.F. Poloni, B. Feltes, D. Bonatto, Melatonin as a central molecule connecting neural development and calcium signaling Funct. Integr. Genomics 11 (2011) 383-388.

[19] M. Weonka, M. Maleszewka, U. Stepinska, M. Markowska, Diurnal differences in melatonin effect on intracellular Ca^{2+} concentration in chicken spleen leukocytes in vitro, J. Pineal Res. 44 (2008) 134-140.

[20] J. Espino, I. Bejarano, S. Paredes, C. Barriga, A. Rodrigues, J. Pariente, Protective effect of melatonin against human leukocyte apoptosis induced by intracellular calcium overload: relation with its antioxidant action, J. Pineal Res. 51 (2011) 195-206.

[21] J. Pariente, C. Camello, P. Camello, GM.Salido, Release of calcium from mitochondrial and nonmitochondrial intracellular stores in mouse pancreatic acinar cells by hydrogen peroxide, J. Membr. Biol. 179 (2001) 27-35.

[22] F. Luchetti, B. Canonico, M. Betti, M. Arcangeletti, F. Pilolli, M. Piroddi, L. Canesi, S. Papa, F. Galli, Melatonin signaling and cell protection function, FASEB J. 24 (2010) 3603-3624.

[23] D.C. Chan, Mitochondrial fusion and fission in mammals, Annu. Rev. Cell Dev. Biol, 22 (2006) 79-99.

[24] K. Itoh, K. Nakamura, M. Iijima, H. Sesaki, Mitochondrial dynamics in neurodegenration, Treds in Cell Biol. 23 (2013) 64-71.

Figure legends

Fig. 1. (A-D) Effects of dexamethasone (DEX) on oxidative stress generation in SH-SY5Y cells. (A-B) ROS production, (C) Glutathione (GSH) and (D) lipid peroxidation were determined in SH-SY5Y cells. (E-F) The effects of melatonin (MEL) attenuate DEX-induced cell death in SH-SY5Y cells. (F) Propidium iodide (PI) fluorescence was used to detect the dead cells. The dead cells were counted and compared to the total numbers of cells present as identified by Hoechst 3342 nuclear staining. (E) The percentages of dead cells are presented in the graph. The values represent the means ± the S.E.M.s of four independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the control-untreated cells, and $^{\#}P < 0.05$ and $^{\#\#\#} P < 0.01$ compared with the DEX-treated cells.

Fig. 2. The effects of melatonin (MEL) on dexamethasone (DEX)-induced increases in cytosolic calcium levels in SH-SY5Y cells. (B) The fluo-4 stained cytosolic calcium is shown in green. (A and C) The changes in cytosolic calcium levels are presented in the graph. The results are expressed as the means ± the S.E.M.s of the three independent experiments. ***P < 0.001 compared with the DEX-treated cells at 0 hr, $**P < 0.01$ compared with the control and $^{\#} P < 0.05$ compared with the DEX-treated cells.

Fig. 3. Effects of melatonin on dexamethasone (DEX)-induced alterations in fission (Drp1 and Fis1) and fusion (Mfn1 and Opa1) protein levels. (A) The SH-SY5Y cells were treated with 1 μM DEX for 1-24 hr. Some cells were pre-treated with 0.25 mM melatonin (MEL) for 1 hr prior to incubation with 1 μM DEX for another (C) 12 hr (Opa1), (C) 18 hr (Mfn1) or (B) 24 hr (Drp1 and Fis1) without changing the culture medium. The control-cultured cells were incubated with culture medium. The alterations in the fission and fusion protein levels were determined with Western blot analyses. Each protein bands was quantified by densitometry, and the differences are represented in the graph as the ratios of the fission and

fusion proteins to the β-actin bands. The values represent the means ± the S.E.M.s of four independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with the control and $^{\#\#}P < 0.01$ compared with the DEX-treated cells.

Fig. 4. The effects of melatonin on dexamethasone-induced morphological alterations of the mitochondria of the SH-SY5Y cells. The SH-SY5Y cells were treated with 1 μM dexamethasone for 24 hr (C-D). The control-untreated cells were incubated with culture medium for 24 hr (A-B). Some cells were treated with 1.0 μM dexamethasone for 24 hr with or without pretreatment with 0.25 mM melatonin for 1 hr (E-F). The mitochondrial morphologies were visualized under a transmission electron microscope. N = nucleus, Scale bars = 1.0 μm and 500 nm.