Melatonin ameliorated okadaic-acid induced Alzheimer-like lesions[1]

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KEY WORDS Alzheimer disease; protein phosphatase; okadaic acid; neurofilament proteins; melatonin

ABSTRACT

AIM: To explore the protective effects of melatonin (Mel) on the abnormal phosphorylation of neuronal cytoskeletal proteins. **METHODS:** We generated a neuroblastoma (SH-SY5Y) cell system in which cytoskeletal proteins are abnormally phosphorylated resulting in microtubule disruption due to the marked inhibition of protein phosphatase activities by okadaic acid (OA). **RESULTS:** OA-induced declines in cell viability and mitochondrial metabolic activity were remarkably prevented by Mel. In addition, the hyperphosphorylation/accumulation of neurofilament-(NF-) H/M subunits and the disruption of microtubules, induced by OA, were significantly inhibited by Mel. **CONCLUSION:** Our results suggest multiple protective functions of Mel against a series of pathological lesions known to culminate in AD, including abnormal phosphorylation of cytoskeletal proteins, microtubule disassembly and mitochondrion-initiated cell toxicity.

INTRODUCTION

The cognitive alterations seen in Alzheimer disease (AD) are associated with widespread neuro-degeneration in the cortex and limbic system. The senile plaques consisting of extracellularly deposited β-amyloid proteins, and neurofibrillary tangles composed of intracellular aggregates of abnormally hyperphosphorylated cytoskeletal proteins are the characterized pathological lesions[1,2]. Therefore, inhibition or reversion of these hallmark lesions is the most attractive strategy for prevention or treatment of the disease. Substantial evidence indicates that melatonin, a pineal hormone has neuroprotective effects in animals and humans, however, the molecular and cellular mechanisms remain unknown. It has been reported that Mel, prevents death of neuroblastoma cells exposed to the β-amyloid peptide[3-5]. Recently, we have also found that Mel effectively inhibits wortmannin-induced hyperpho-sphorylation of tau in rat brain[6]. The multiple anti-neurodegenerative effects of estrogen, such as enhanced neuronal activities related to learning and memory and reduced β-amyloid burdens both *in vitro* and *in vivo*, have also been well documented[7-9]. In this study, we have further invistigated the effect of Mel, on okadaic acid (OA), a potent protein phosphatase (PP)-2A and PP–1 inhibitor, induced lesions in neuroblastoma cells.

[1] This work was supported in part by grants from the National Natural Science Foundation of China (39925012, 30170221 and 30100057), and the Science and Technology Committee of China (G1999054007).
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Received 2003-03-11 Accepted 2003-09-09

MATERIALS AND METHODS

Cell culture Human neuroblastoma SH-SY5Y cells obtained from Prof K IQBAL (NYS Institute for Basic Research, Staten Island, NY, USA) were distributed in Dulbecco's modified Eagle's medium (DMEM) with 5 % fetal bovine serum and benzylpenicillin/streptomycin (5 % CO_2 and 95 % air)[1].

Cell viability Cell viability and mitochondrial activity were determined by colorimetric assay of crystal violet[10] and modified 5-diphenyl tetrazolium bromide (MTT)[11,12], respectively. Different concentrations of melatonin (Mel, Sigma, St Loius, MO, USA) were used to treat the cells for 24 h prior to OA treatment or simultaneously with OA treatment. Each experiment was repeated at least 3 times with octuple (n=24). The protein concentration was measured by a modified Lowry method[13].

Immunocytochemistry For immunocytochemistry, cells were plated at a density of 1.0×10^5 cells/cm^2 on glass coverslips and fixed for 10 min in 4 % paraformaldehyde in 1×phosphate-buffered saline (PBS) pre-warmed at 37 ºC for 10 min. Fixed cells were washed 3 times in PBS containing 0.1 % Triton X-100 and incubated with 10 % goat serum, 3 % BSA, and 2 % Tween-20 in PBS for 1 h at room temperatures to prevent nonspecific binding of antibodies. The primary antibodies SMI31-34 (1:5000 dilution; Sternberger Monoclonals Inc, Lutherville, ML, USA) and DM1A (1:500 dilution; Sigma, St Loius, MO, USA) were added and incubated overnight at 4 ºC. The slides were developed by biotinylated secondary antibodies (1:200) and avidin-peroxidase conjugate (1:200)/Diaminobenzidine (0.05 %) (ZEMED, Beijing, China). The developed slides were mounted with glycerol. The immunostaining was quantified using Kodak Digital Science 1D software (Cold Spring Harbor Inc., Beijing, China). At least 500 cells were computed and analyzed in each experiment.

Statistical analysis Data were expressed as mean±SD. Statistical analysis was performed with One-Way ANOVA, followed by LSD's *post hoc* tests, which was provided by SPSS 10.0 statistical software. Statistical significance was accepted at the level of $P<0.05$.

RESULTS

Mel attenuates OA-induced decrease in cell viability and mitochondrial activity Exposure of the cells to OA 15 nmol/L for 24 h induced increased cell death, as determined by crystal violet assay. Under the same condition, a striking decrease in mitochondrial metabolic activity was observed by using MTT measurement. Remarkable reversion in both cell viability and mitochondrial activity was achieved when 50 μmol/L or 100 μmol/L, but not 500 μmol/L of Mel was added during or prior to the OA treatment (Fig 1, 2).

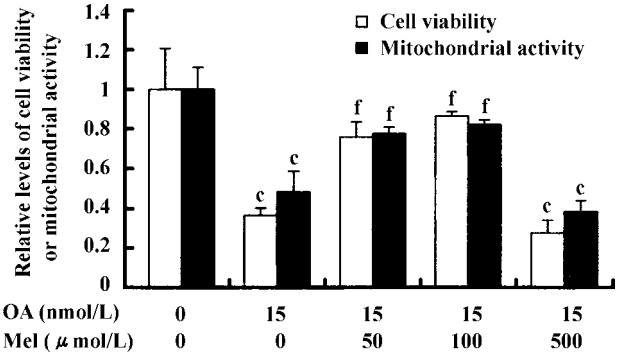

Fig 1. Effects of melatonin (Mel) on OA-induced cell viability and mitochondrial activity when added simultaneously with OA. SH-SY5Y cells were treated without (control), or with OA in the presence of different concentrations of Mel for 24 h. n=24. Mean±SD. $^cP<0.01$ *vs* control; $^fP<0.01$ *vs* OA 15 nmol/L treatment.

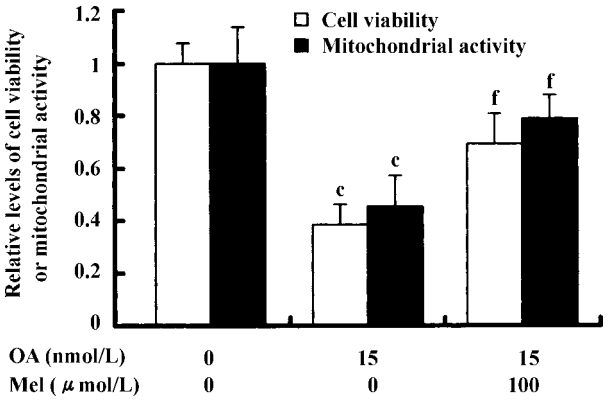

Fig 2. Effects of Mel on OA-induced cell viability and mitochondrial activity when added prior to OA treatment. SH-SY5Y cells were pretreated with indicated concentrations of Mel prior to incubation with OA. n=24. Mean±SD. $^cP<0.01$ *vs* control; $^fP<0.01$ with respect to 15 nmol/L OA treatment.

Mel attenuates OA-induced hyperphosphorylation of neurofilament To illustrate the possible mechanism of Mel in the cytoprotection observed in the present study, we studied the effect of Mel on OA-induced phosphorylation of neuronal cytoskeletal protein neurofilament (NF). It was found by immunocy-

tochemistry that phosphorylated (p)-NF-H/M recognized by antibody SMI31 was evenly distributed in the cell processes and cell surface before OA treatment. After incubation with OA 15 nmol/L, the staining was condensed in the proximal end of the cell processing with an increased staining in the cell body. Quantitative analysis revealed a significant increase in the level of NF-H/M of SMI31. Mel notably inhibited the phosphorylation and accumulation of NF (Fig 3). A similar result was obtained when another phosphorylation de-

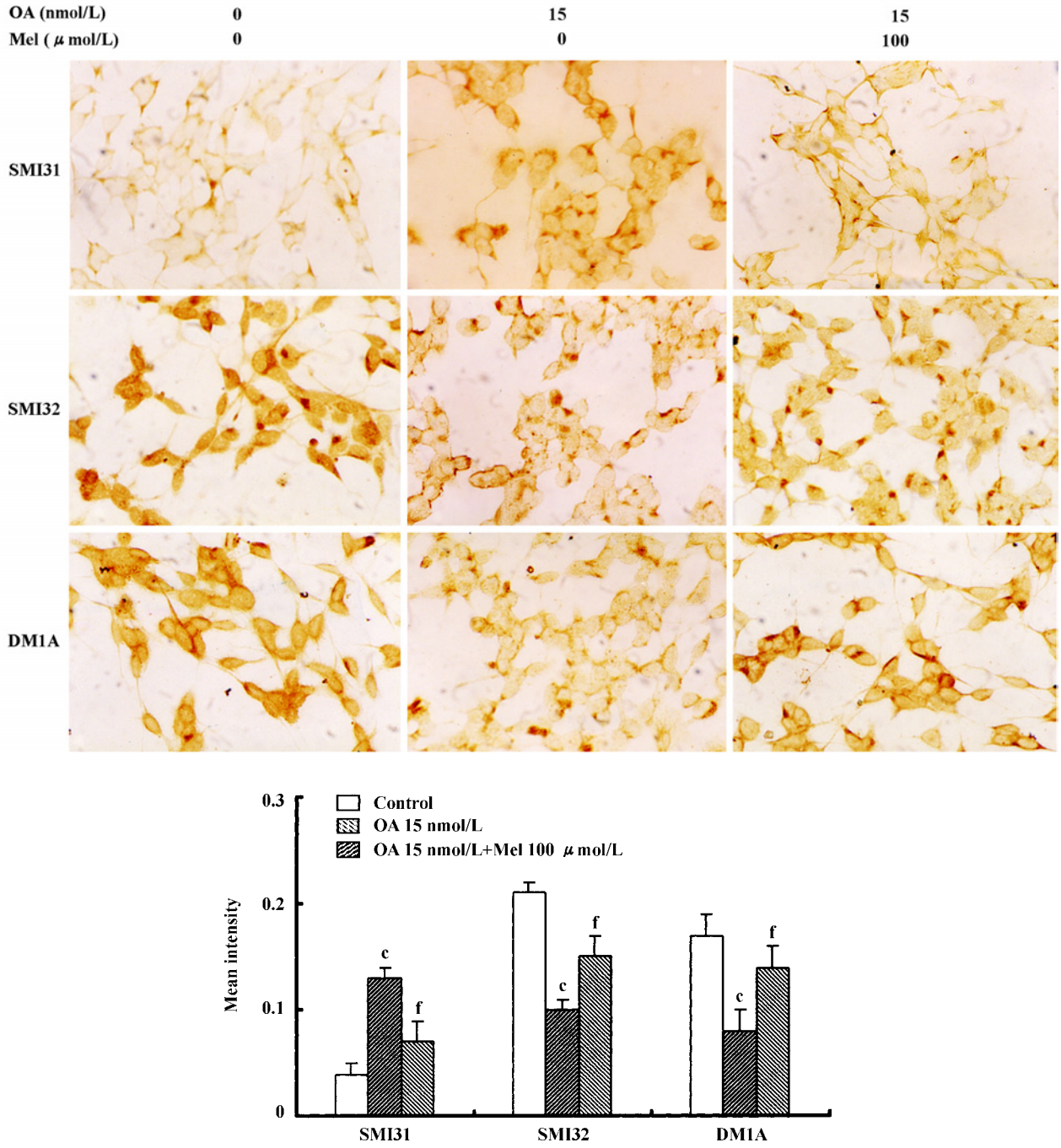

Fig 3. Effects of Mel on OA-induced Alzheimer-like impairment in neuronal cytoskeletal proteins. SY5Y cells were incubated in the absence or presence of 15 nmol/L OA alone or of 15 nmol/L OA and 100 mmol/L Mel. After the treatment, cells were immunostained with SMI31, SMI32, DM1A for phosphorylated NF-H/M, non-phosphorylated NF-H/M, or tubulin, respectively. Mel effectively arrests OA-induced hyperphosphorylation and accumulation of NF, and the disruption of microtubule network. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* OA 15 nmol/L.

pendent antibody SMI34, instead of SMI31, was used (not shown).

On the other hand, the immunostaining of non-phosphorylated (np)-NF-H/M specifically recognized by antibody SMI32 was higher in cell body and cell processes under normal conditions. After exposure of the cells to OA 15 nmol/L for 24 h, the staining in the cell body became weaker and the condensed spots were seen in the proximal ends of the cell. A significant preservation of SMI32 staining was observed when Mel 100 μmol/L was added along with OA 15 nmol/L to the culture medium. Quantitative analysis of the data obtained from the immunocytochemistry demonstrated that Mel considerably unblocked phosphorylation of NF-H/M induced by the inhibitor (Fig 3). A similar immunocytochemical profile was observed using SMI33, another antibody that reacts with a non-phosphorylated epitope of NF-H/M (not shown).

Mel attenuates OA-induced disruption of the microtubule In addition to NF proteins, immuno-staining for tubulin (using antibody DM1A) was significantly decreased after OA treatment, suggesting a disruption of the microtubule network due to the inhibition of PP-2A and PP-1. Mel significantly reduced OA-induced microtubule breakdown (Fig 3).

Mel attenuates OA-induced drastic changes in cell morphology Treatment of SY5Y cells with OA also caused drastic changes in cell morphology. Incubation of the cells with OA 15 nmol/L induced retraction of cell processes and formation of cell clusters; and cells became round, shrunk and aggregated after incubation with OA 30 nmol/L for 24 h. A partial cytoprotection was observed when Mel 50 μmol/L or 100 μmol/L was added to the culture medium along with OA 15 nmol/L (data not shown).

DISCUSSION

Abnormal phosphorylation of various cytoskeletal proteins is the key step in the formation of neurofibrillary tangles, and represents a hallmark lesion seen in the AD brain. Deficiency in protein phosphatase activity and oxidative stress are involved in the pathogenesis of the disease [14]. Using Mel to prevent declines in protein phosphatase activities may be an effective intervention in AD. In this study, we made an Alzheimer-like protein phosphatase deficient model by culturing neuroblastoma cell with OA, and investigated the protective effect of Mel. We found that Mel was able to prevent the OA-induced declines in cell viability and mitochondrial metabolic activity. In addition, the hyperphosphorylation of neurofilament- (NF-) H/M subunits and the disruption of microtubules, induced by OA, were significantly inhibited by Mel.

Mel is a pineal secreted hormone. It has been well documented that Mel is involved in the aging process and decreased secretion of the Mel has been found during aging with a more profound reduction reported in populations of AD [15-17]. The cytoprotective effect of Mel may also be attributed to its free radical scavenger or antioxidant properties[18-20].

The phenomenon of OA-induced inhibition of PP activity[21] and oxidative stress[22], and the fact that antioxidants can prevent protein hyperphosphorylation in cell culture[23] has been well described. In the present study, we studied the effect of Mel on neuronal cytoskeletal proteins, such as NF phosphorylation and microtubule disruption induced by inhibition of PP-2A and PP-1. NFs are composed of three protein subunits, namely NF-H, NF-M, and NF-L, with apparent molecular mass of about 200 kDa, 160 kDa, and 70 kDa, respectively. Recently, we have found that phosphorylated (p)-NF-H/M is significantly elevated in the brain[24] and cerebrospinal fluid[25] of AD patients. Based on the observation that Mel prevents abnormal phosphorylation of NFs and thus microtubule disassembly, we speculate that the possible mechanism of Mel in preventing OA-induced cytotoxicity observed in this study may be through its antioxidative stress properties, and this may provide a way to correct mitochondrial dysfunction and cytoskeleton disruption. Further studies are needed to illustrate the detailed mechanism of Mel in these protective processes, especially in remedying the deficit in phosphatase activity. Since Mel has been reported to be nontoxic and permeable to the blood-brain barrier, it may, therefore, be a prime candidate for therapy of AD.

ACKNOWLEDGEMENT We are grateful to Dr Hua-xi XU (The Rockefeller University, New York, NY, USA) for critical reading of the manuscript and helpful discussion. We also thank Dr Khalid IQBAL and Inge GRUNDKE-IQBAL (NYS Institute for Basic Research, Staten Island, NY, USA) for comments and discussion.

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