Role of Melatonin in Reducing Amphetamine-Induced Degeneration in Substantia Nigra of Rats via Calpain and Calpastatin Interaction

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ABSTRACT: Excessive intracellular calcium levels induce calpain activation, thereby triggering the cell death cascade. Several lines of evidence have demonstrated the neuroprotective role of the overexpression of calpain inhibitor, calpastatin. In this study, amphetamine-induced degeneration in the substantia nigra of rats was determined by evaluating the decrease in the levels of tyrosine hydroxylase phosphorylation. Amphetamine significantly decreased calpastatin levels but increased calpain levels. An induction in calpain activity was demonstrated by an increase in the formation of calpain spectrin breakdown products. The deleterious effects of amphetamine exposure were diminished in rats by pretreatment with melatonin. In addition, the effect of melatonin on calpastatin expression was investigated in human neuroblastoma SH-SY5Y cells. Melatonin was able to increase the calpastatin levels, and this effect could be blocked by luzindole, a melatonin receptor antagonist. These results demonstrate the neuroprotective ability of melatonin and its role in inducing calpastatin expression via a receptor-dependent pathway.

KEYWORDS: Amphetamine, calpain, calpastatin, melatonin, rat brain, neuroprotection

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Introduction

Amphetamine is a psychostimulant that enhances the release of dopamine (DA) from synaptic vesicles and thereby enhances the amount of DA in the central nervous system. Several studies have demonstrated that excessive DA levels and subsequent DA autooxidation can result in the formation of reactive oxygen species (ROS), which may promote neuronal degeneration. Amphetamine and its derivatives such as methamphetamine can cause damage to nigrostriatal dopaminergic nerve terminals and neurons, causing symptoms similar to those observed in neurodegenerative diseases such as Parkinson disease. The neurotoxicity of amphetamine leads to the depletion of DA, tyrosine hydroxylase (TH), and the loss of the DA transporter. It has been demonstrated that amphetamine-induced nerve terminal and neuronal cell death are caused by multiple pathways, including the inhibition of electron transport chain activity in mitochondria, a decrease in the antiapoptotic Bcl-2–related protein, an increase in proapoptotic proteins (BAX, BAD, and BID), and the activation of cysteine proteases or the caspase death pathway. Another regulator of the cell death process is calpain activation. Calpain is a calcium (Ca²⁺)-dependent nonlysosomal cysteine protease enzyme. Calpain activity is associated with disturbed calcium levels, and it is regulated by its inhibitor, calpastatin. Calpastatin is the only known inhibitor of calpain, which competitively binds at the Ca²⁺-binding site of calpain.

Many studies have reported that calpastatin protein levels and activity decrease in various injury models. Moreover, the overexpression of calpastatin prevents oxidative stress–induced neuronal degeneration and the behavioral deficits associated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment in mice. Related to the neuroprotective capacity of calpastatin, it has been documented that melatonin is able to restore calpastatin levels and reduce calpain and caspase activation in neuroblastoma SH-SY5Y cells. Therefore, the aim of this study was to investigate the importance of the calpastatin and calpain interaction and the potential role of melatonin as a neuroprotective agent for the calpain–dependent death processes in the substantia nigra of d-amphetamine–treated rats.

Materials and Methods

Drugs and chemicals

d-amphetamine hydrochloride and melatonin were purchased from Sigma-Aldrich (St Louis, MO, USA). The rabbit polyclonal anti-TH phospho-Ser40 (TH-pSer40) antibody and the mouse monoclonal antispectrin and actin antibodies were purchased from Chemicon International, Inc. (Temecula, CA, USA). The rabbit polyclonal anticalpain (H240) and anticalpastatin (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The rabbit polyclonal anti-TH phospho-Ser40 (TH-pSer40) antibody was purchased from Promega (Madison, WI, USA). The rabbit polyclonal anti-calpain (H240) and anticalpastatin (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

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Biotechnology, Inc. (Santa Cruz, CA, USA). The antirabbit IgG horseradish peroxidase (HRP)–linked, and antimouse IgG (H&L) HRP-linked antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The protease inhibitor cocktail set I and phosphatase inhibitor cocktail set II were obtained from Calbiochem (Darmstadt, Germany). Enhanced chemiluminescence Western blot detection reagents (ECL Plus) were purchased from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals used in this study were of analytical grade and were obtained from either Sigma-Aldrich or Lab-Scan Analytical Science (Dublin, Ireland).

**Animals**

Wistar rats were obtained from the National Experimental Animals Center of Mahidol University, Salaya Campus, Thailand. The animals had free access to water and food and were maintained in a 12 hours light/dark cycle (lights on at 7:00 AM). Female rats were placed with a male in a hanging wire cage for a period of 2 weeks, after which time the females were singly housed in poly-carbonate cages. Embryonic day 0 (E0) was designated as the day a sperm plug was detected. Beginning on E22, the presence of a litter was checked in the morning and afternoon and the day of birth was designated as postnatal day 0 (P0). A total of 12 litters (n = 5–6 per group) were used as the subjects for this experiment. All animal procedures were approved by the Laboratory Animal Care and Use Committee of Mahidol University.

**Drug administration**

Rat pups were randomly assigned to either repeated saline or d-amphetamine groups. The animals were injected subcutaneously with saline or d-amphetamine once daily (10:00 AM) for 7 consecutive days, starting on postnatal day 4 (P4), using an increasing dose of d-amphetamine: days 1 to 2, 5 mg/kg; days 3 to 7, 10 mg/kg. Melatonin–pretreated rats were injected with melatonin (2 mg/kg, dissolved in saline) subcutaneously 30 minutes prior to d-amphetamine administration. The animals were killed on P10 and the brains were immediately removed and dissected under a stereo dissecting microscope at 4°C. Substantia nigras were dissected and stored at −80°C until used. The nigral tissue was homogenized in radioimmunoprecipitation assay buffer (50-mM Tris-HCl pH: 7.4, 150-mM NaCl, 1-mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% protease inhibitor) and then centrifuged at 12,000 g for 15 minutes at 4°C. The protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) and normalized to the levels of actin expression within the same sample.

**Western blot analysis**

The protein solutions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred to nitrocellulose membranes. After the transfer, the nitrocellulose membrane was washed with Tris-buffered saline (TBS) for 5 minutes. The membrane was incubated with blocking solution (5% nonfat dry milk in 0.1% Tween-TBS (TBST)) and washed 3 times for 5 minutes, each time with TBST. The membrane was incubated with the primary antibodies at 4°C overnight. After incubation, the membrane was washed 3 times for 5 minutes each time with TBST, incubated with HRP-conjugated secondary antibody for 1.5 hours and then washed 3 times for 5 minutes each time with TBST. Finally, the membrane was visualized using chemiluminescent ECL Plus-Western blotting detection reagents and exposed on x-ray film. Immunoblot bands were quantified by measuring the density of each band using a densitometer with Scion imaging program (National Institutes of Health, Bethesda, MD, USA). All data were normalized to the levels of actin expression within the same sample.

**Statistical analysis**

Data were expressed as the mean ± SEM. Significance was assessed by 1-way analysis of variance (ANOVA) followed by Tukey-Kramer test using the scientific statistic software SPSS version 16. Probability (P) values less than .05 were considered to be statistically significant.

**Results**

**Effect of melatonin on d-amphetamine–induced reduction in TH phosphorylation and calpastatin in substantia nigra of rat**

Western blot analysis was used to determine whether repeated d-amphetamine administration would alter the levels of phosphorylation of serine 40 of TH (TH-pSer40) and calpastatin in the substantia nigra of rat. The results show that d-amphetamine administration caused a significant decrease in the levels of TH-pSer40 (Figure 1) and calpastatin (Figure 2) in the substantia nigra of rat brain when compared with control-untreated rat.
TH-pSer40 and calpastatin protein levels in the substantia nigra decreased to 77% ± 1.4% and 72% ± 1.6% of the control value, respectively. Pretreatment with melatonin prior to the administration of d-amphetamine significantly increased the levels of TH-pSer40 (Figure 1) and calpastatin (Figure 2) in the substantia nigra to 89% ± 2% and 89% ± 0.6% of the control value compared with the d-amphetamine–treated rat, respectively. Melatonin had no effect on the levels of TH-pSer40 (Figure 1) and calpastatin (Figure 2) in the substantia nigra of rat brain when compared with control-untreated rat.

**Effect of melatonin on d-amphetamine–induced increase in the amount and activity of calpain in substantia nigra of rat**

The effect of melatonin on the d-amphetamine–induced increase in the amount and activity of calpain was determined in the substantia nigra of rat brain using Western blot analysis. d-amphetamine administration caused a significant increase in the amount of calpain (Figure 3A) and calpain SBDP-145 (Figure 3B) to 193% ± 27.1% and 162% ± 20.3% of the control value when compared with control-untreated rats, respectively. Pretreatment with melatonin prior to the administration of d-amphetamine significantly decreased the amount of calpain (Figure 3A) and calpain SBDP-145 (Figure 3B) in the substantia nigra to 128% ± 12.2% and 101% ± 12.8% of the control value when compared with the d-amphetamine–treated rat, respectively. Melatonin alone had no effect on the amount of calpain (Figure 3A) and calpain SBDP-145 (Figure 3B) in the substantia nigra of rat brain when compared with control-untreated rats, respectively.

**Effect of melatonin on calpastatin levels in SH-SY5Y cells**

Melatonin-treated SH-SY5Y cells at melatonin concentrations between 0.001 and 1.0 mM for 24 hours had no effect on cell viability when compared with control-untreated cells (data not shown). The levels of calpastatin in SH-SY5Y cells were investigated with a specific antibody using Western blot analysis. Melatonin at various concentrations (0.001, 0.05, 0.125, 0.25, and 0.5 mM) was then added to SH-SY5Y cells and incubated for 24 hours. The results showed that the calpastatin level in SH-SY5Y cells was significantly increased after incubation with melatonin at 0.125 mM (133% ± 8.5% of control values) when compared with control-untreated cells. The calpastatin level in SH-SY5Y cells after incubation with melatonin at 0.001, 0.05, 0.25, and 0.5 mM concentrations was 93% ± 6.4%, 110% ± 8.7%, 113% ± 10.4%, and 95% ± 7.8% of the control values, respectively (Figure 4).

**Effect of luzindole on melatonin–induced increase in calpastatin levels in SH-SY5Y cells**

SH-SY5Y cells were treated with 0.125–mM melatonin for 24 hours or 1–μM luzindole for 1 hour. Some cells were incubated with 1.0–μM luzindole for 1 hour and then followed by...
incubation with 0.125-mM melatonin for another 24 hours. The control-cultured cells were incubated with culture medium for 24 hours. Exposure to luzindole at 1-μM for 1 hour did not change calpastatin levels (92% ± 8.0% of control values) when compared with control-untreated cells. Melatonin-treated cells at 0.125 mM for 24 hours exhibited significantly increased calpastatin levels (116% ± 3.1% of control values) when compared with control-untreated cells. Pretreatment of the cells with luzindole significantly decreased the calpastatin levels (98% ± 3.0% of control values) when compared with melatonin treatment without luzindole pretreatment (Figure 5).

Discussion
The results of this study demonstrated that amphetamine is able to induce degenerative process in the substantia nigra of rats, which is observed by the reduction in the levels of TH phosphorylation. Previous study has demonstrated that repeated amphetamine administrations produce a long-lasting decrease in striatal TH level. Amphetamine has been considered to induce neurotoxicity by enhanced DA release via exchange diffusion, reverse transport, and channel-like transport phenomena. Dopamine is auto-oxidized, which causes free radical formation leading to neuronal damage or death. The toxicity of amphetamine can cause neuronal apoptosis through the mitochondrial, endoplasmic reticulum, and receptor-mediated death pathways. At the mitochondrial level, amphetamine leads to enhanced ROS formation and increased mitochondrial dysfunction. Mitochondrial dysfunction leads to a leakage of mitochondrial Ca$^{2+}$ into the cytosol, and high levels of intracellular Ca$^{2+}$ may contribute to the activation of calpain. Recent evidence has shown that cytosolic calcium levels are increased in amphetamine-induced toxicity in neuroblastoma SH-SY5Y cells.

Calpain belongs to a large family of calcium-dependent cysteine proteases that are ubiquitously distributed in the mammalian brain. Calpain activation can cleave caspase 3 into its active forms as well as cleave caspase, which can in turn activate caspase 3-induced apoptosis. Several studies have shown the significance of increased calpain activity on neurodegeneration. Pathological calpain activation is triggered by elevation of the intracellular calcium concentration. The activation of calpain can be detected by immunoblot analysis by monitoring the formation of calpain-specific SBDP at 145 kDa. There is strong evidence linking ROS-initiated oxidative damage to loss of intracellular Ca$^{2+}$ homeostasis and
Our previous studies have demonstrated that melatonin-treated cells.

The overexpression of calpastatin can attenuate amyloid β-peptide–induced calpain activation and toxicity in PC12 cells. Calpastatin overexpression can also block kainic acid–induced cytoskeletal protein disruption, extracellular-regulated kinase activation, and neurodegeneration in mice. In this study, the rescue effect of melatonin to reduce calpain activation was observed in amphetamine-treated rats, and our present findings are consistent with previous studies that demonstrated that melatonin decreases inflammation and calpain expression in spinal cord injury in rats. Treatment with melatonin also reduces the neurotoxic effects of MPP⁺ (1-methyl-4-phenylpyridinium) on induction in calpain activity, activation of the cleavage of cdk5/p35 to cdk5/p25, and increases in the apoptotic cell population in CGN cultures. Furthermore, melatonin can reduce calcium overload from mitochondria and block mitochondrial permeability transition pore–dependent cytochrome c release and caspase 3 activation.

In conclusion, this study demonstrated that amphetamine-induced degenerative process in the substantia nigra of rats occurs partly via calpain activation. Melatonin can reverse the amphetamine-induced neurotoxicity. By exploring the functional role of melatonin related to calpastatin, we found that melatonin regulates the increase in calpastatin levels through a receptor-dependent mechanism. The neuroprotective effect of melatonin related to calpastatin, we found that melatonin decrease TH phosphorylation and calpastatin levels but increases calpain activation in the substantia nigra of rats. Melatonin provides a protective effect against the amphetamine-induced calpain-dependent death pathway in the substantia nigra, demonstrated by the ability of melatonin treatment to restore TH phosphorylation and calpastatin levels, as well as reduce the activation of calpain. The protective effects of melatonin are likely derived from its antioxidant property, which reduces amphetamine-induced oxidative stress in neuronal cells and then countered the effect of oxidative stress-induced loss of intracellular Ca²⁺ homeostasis and calpain activation. In addition, we found that melatonin treatment increased calpastatin levels in neuroblastoma SH-SY5Y cells. Moreover, the increase in calpastatin levels after melatonin treatment is via a receptor-dependent mechanism according to whether pretreatment of the cells with luzindole (a melatonin receptor antagonist) was able to abolish the effect of melatonin–induced increase in calpastatin levels. It appears that the changes in calpastatin levels and cell viability are inversely correlated with calpain activation. It is also known that the calpastatin/calpain ratio is an important factor in the regulation of calpain activity within the cell, and the overexpression of calpastatin can attenuate amyloid β-peptide–induced calpain activation and toxicity in PC12 cells. Calpastatin overexpression can also block kainic acid–induced cytoskeletal protein disruption, extracellular-regulated kinase activation, and neurodegeneration in mice.

**Figure 5.** The effect of luzindole on the melatonin-induced increase in calpastatin levels in SH-SY5Y cells. Cells were treated with 0.125-mM melatonin (MEL) for 24 hours or 1-µM luzindole for 1 hour. Some cells were pretreated with 1.0-µM luzindole (LUZ) for 1 hour and then followed by treatment with 0.125-mM melatonin for another 24 hours. The control-untreated cells were incubated with culture medium for 24 hours. The calpastatin levels were determined using Western blot analysis. The results are expressed as the mean ± SEM of the 4 independent experiments. *P < .05 when compared with control-untreated cells; †P < .05 when compared with melatonin-treated cells.

Calpain activation. The activation of calpain occurs after an early wave of oxidative damage at the peak of mitochondrial dysfunction. The mitochondrial damage may result in calcium overload within the cells, inducing the nuclear translocation of proapoptotic factors such as endonuclease G (EndoG) and apoptosis-inducing factor (AIF) from mitochondria. Moreover, calpain activity is also regulated by calpastatin, an endogenous calpain inhibitor that functions as an inhibitor by binding with high affinity and strict specificity to calpain. The ratio of calpastatin to calpain is important for the control of calpain activity, and it has been demonstrated that calpastatin-containing fractions extracted from nerve growth factor–treated PC12 cells can decrease calpain activity in PC12 cells. The overexpression of calpastatin in cultured cerebellar granule neurons (CGNs) can inhibit calcium-activated calpain, prevent calcium overload, and rescue neurons from excitotoxicity. The overexpression of calpastatin has also been shown to exhibit neuroprotective ability and is able to attenuate the MPTP-induced loss of nigral DA neurons in the mouse and the release of AIFs in ischemic neuronal injury. Our previous studies have demonstrated that melatonin can attenuate methamphetamine-induced nigrostriatal degeneration in rats and methamphetamine-induced calpain-dependent cascades and degeneration in neuroblastoma SH-SY5Y-cultured cells. In this study, it was revealed that
contribution to the acquisition and analysis of the data. PG contributed to the analysis and interpretation of data. BC contributed to the experimental design, analysis, and interpretation of the data and critical review of the manuscript. All authors reviewed and approved the final manuscript.

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