Original Article Involvement of Bcl-2-associated athanogene (BAG)-family proteins in the neuroprotection by rasagiline

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Received August 11, 2015; Accepted October 7, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Rasagiline, a novel monoamine oxidase (MAO)-B inhibitor, has a mild to moderate effect in relieving Parkinson's disease (PD) symptoms as well as unique neuroprotective effects. Previous studies demonstrated rasagiline protect neurons by regulating Bcl-2 family proteins. Our study aimed to study whether Bcl-2-associated athanogene (BAG)-family proteins, which were reported closely associated with neurodegenerative disease, were involved in the neuroprotective effect of rasagiline. We found that after the administration of 1-methy1-4-phenvl-1,2,3,6-tetrahvdropvridine (MPTP), BAG2 and BAG5 proteins were up-regulated in the substantia nigra dopaminergic neurons of PD mouse model. A further increase of BAG2 and BAG5 was detected after intragastric administration of rasagiline to post-MPTP lesioned mice. Thus, the current study proved the association of BAG family proteins with PD, and suggested the involvement and a positive role of BAG2, BAG5 in the neuroprotection of rasagiline. These preliminary results implicate a novel pathway for further study on neuroprotection of rasagiline.

Keywords: Rasagline, BAG protein, MPTP, neuroprotection

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorder affecting 1.7% of the population above 65 years of age in China [1]. It is pathologically characterized by loss of dopaminergic neurons in the substantia nigra (SN) pars compacta accompanied by the presence of Lewy bodies in residual dopaminergic neurons. The etiology of PD involves the genetic predispositions, environmental exposures and the gene-environment interactions. There is growing evidence that oxidative stress, mitochondrial dysfunction, altered protein handling, calcium disbalance and neuroinflammation were involved in the pathogenesis of the disease [2].

Unfortunately, as a progressive disorder, the classical motor features of PD become clinically apparent when there is over 60% dopamine depletion in the nigrostriatal pathway. Current dopamine replacement therapy is only symptomatic. Therefore, major research efforts have focused upon developing neuroprotective interventions with the aim to slow the neurodegeneration and disease progression [3]. Rasagiline (N-propargyl-1-(R)-aminoindan), a novel, highly potent irreversible monoamine oxidase (MAO)-B inhibitor, was one of the most promising candidate neuroprotective drugs. Clinical trials of rasagiline in patients with Parkinson's disease suggest that rasagiline has a mild to moderate effect in relieving PD symptoms as well as some disease-modifying effects [4]. Basic research using cellular and animal models found that the MAO-B inhibitors protect dopaminergic neurons through several pathways including stabilization of mitochondrial membrane and induction of antipoptotic Bcl-2 protein family [5-8]. As a multi-targets drug, detailed mechanism on the



Figure 1. Expression of BAG1, BAG2, BAG3, BAG5 protein in the SNpc of MPTPlesioned mice. A. Representative Western blotting of TH, BAG proteins and GAPDH. A up-regulation of BAG2 and BAG5 in SNpc after MPTP treatment was indicated. B. The histogram represents the MPTP group and the control group. The ratio of TH, BAG proteins to GAPDH in intensity (OD value) was regarded as the protein level for a sample. Data are expressed as the mean \pm SD (n=3). *P<0.05, compared with the control group.

interactions of MAO-B inhibitors with the target proteins was not fully clarified.

Bcl-2-associated athanogene (BAG)-family proteins were originally identified in the study of the anti-apoptosis protein Bcl-2. The BAG proteins are an evolutionarily conserved family proteins characterized by a common conserved region located near the C terminus termed the BAG domain but differ markedly in their N-terminal regions. In humans, there are currently six family members identified, designated BAG1 to BAG6. Considered as co-chaperones with a variety of binding partners, BAG proteins are multifunctional proteins that participate in diverse cellular processes such as stress signaling, neuron differentiation, and cell cycle [9]. The interactions of BAG1,2,3,5 with Hsc70/Hsp70 or CHIP demonstrated a link between the BAG family proteins and the UPS and autophagy protein degradation pathways [9, 10]. Our previous work found after 1-methyl-4-phenylpyridinium (MPP⁺) treatment in PC12

cells, BAG5 was up-regulated which inhibited apoptosis by increasing the expression of anti-apoptotic proteins, including Bcl-2 and Bcl-xl [11]. Otherwise, we found that both BAG2 and BAG5 stabilized PINK1 by decreasing the ubiquitination of PINK1, and BAG5 protects against mitochondrial oxidative damage through regulating PINK1 degradation [12, 13]. These studies indicated that the BAG family proteins were associated closely with neurodegenerative disease and possibly play an antiapoptotic role against neurotoxicity.

In the present study, we sought to examine whether BAG proteins levels were changed in dopaminergic neurons of MPTP mouse model. We also sought to investigate the possible role of BAG proteins in the neuroprotection by rasagiline.

Materials and methods

Animal grouping and MPTP model

All studies were carried out in accordance with the Guidelines for Animal Experiments of Central South University, Changsha, China. Sixweek-old, male, C57BL/6 mice (Hunan, China), weighing 20 to 25 g, were randomly divided into two groups of 20 mice, 2-3 mice each cage. Drinking water and food were supplied ad libitum to each cage. A 2-wk period was allowed for acclimatization. Then they were subjected to a subacute MPTP regimen. Each group received intraperitoneal injections of MPTP or vehicle at the concentration of 25 mg/kg once daily for 10 consecutive days, followed by a further 3 days resting period. Assessment of SNpc was performed 13 days after the start of the MPTP administration.

Two additional animal groups (MPTP/Rasagiline and MPTP/water) were set by the same criteria



Figure 2. The up-regulation of BAG2 and BAG5 in SNpc after MPTP treatment shown by immunohistochemistry. A. MPTP-treated mice were transcardially perfused, then their brains were fixed for immunohistochemistry. Dopaminergic neurons, as identified by immunostaining against TH (red) expressed BAG2, BAG5 (red) (100×). Expression of BAG2, BAG5 increased after MPTP treatment. B. The histogram represents the MPTP group and the control group. TH-positive, BAG2-positive, BAG5-positive cells were quantified across selected MPTP-sensitive sections. Data are expressed as the mean \pm SD (n=3). *P<0.05, compared with the control group.

above for further study (n=20, each group). In this paradigm, MPTP (25 mg/kg, i.p., per day) was administrated for 10 days, followed by another 3 days resting period. At day 14, either rasagiline (0.08 mg/kg, p.o.) or water were intragastric administered for 10 days, reaching a total treatment period of 23 days.

Sample preparation

After all those administrations above, half of those mice from each group were deeply anaesthetized with 10% chloral hydrate and transcardially perfused with 50 ml 4% paraformaldehyde buffered with phosphate buffer solution (PBS, 0.01 M, pH 7.14). The whole brain was then rapidly removed from the skull, coronal sections of 10 µm each were cut onto poly-L-lysine coated slides at -20°C using a freezing microtome, and the sections of nigral formation were employed to perform the immunohistochemistry.

Remaining mice of each group were decapitated and the ventral midbrain were rapidly dissected and homogenized in ice cold-lysis buffer containing a protease inhibitor cocktail to obtain whole cell protein. Lysates were cleared by centrifugation and protein concentration was determined by BCA kit.

Immunohistochemical staining

After washing six times with 0.01 M phosphate buffer saline (PBS, pH 7.2), brain sections were incubated with 5% fetal bovine serum in 0.01 M phosphate buffered saline containing 0.2% Triton X-100 for 30 min at room temperature and then washed and for 30 min at room temperature

and incubated with primary antibodies against Tyrosine Hydroxylase (TH) (Rabbit polyclonal, 1:800, abcam, USA), BAG2 (Rabbit polyclonal, 1:50, abcam, USA), BAG5 (Rabbit polyclonal, 1:500 Rabbit, abcam, USA) in 0.01 M PBS, at 4°C overnight. Then sections were washed in



Figure 3. Effect of the rasagiline on the expression of TH, BAG2, BAG5 in SN of MPTP-lesioned mice. MPTP-lesioned mice treated with rasagiline or water were designated as MPTP/Rasagiline group and MPTP/Water group. A. Representative Western blotting of TH, BAG2, BAG5 and GAPDH. Post-MPTP treatment with rasagiline markedly increased the BAG2, BAG5 level of dopaminergic neurons in the MPTP/Rasagiline group. B. The histogram represents the MPTP/Rasagiline group and MPTP/Water group. The ratio of TH, BAG2, BAG5 protein to GAPDH in intensity (OD value) was regarded as the protein level for a sample. Data are expressed as the mean \pm SD (n=3). *P<0.05, compared with the MPTP/Water group.

0.01 M PBS for 30 min at room temperature. The second antibodies were goat anti-rabbit or anti-mouse IgG conjugated to cy3 (1:200, Vector Laboratories, Burlingame, CA, USA). After incubation with second antibodies for 2 h, sections were again washed in PBS, mounted in an anti-fading reagent (Vectashield, Vector Laboratories) under a coverslip. Images were captured on a fluorescence microscope (Nikon, Japan).

Western blotting

Equal amounts of proteins were fractionated by SDS-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane. The membranes were blocked with 5% defatted milk in TBS-Tween (TBS-T) (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) and incubated with antibodies against TH (Rabbit polyclonal, 1:200, abcam, USA), BAG1 (Rabbit polyclonal,

1:300, abcam, USA), BA-G2 (Rabbit polyclonal, 1:500, abcam, USA), BA-(Rabbit polyclonal, G3 1:800, abcam, USA), BA-(Rabbit polyclonal, G5 1:700 Rabbit, abcam, USA), GAPDH (mouth monoclonal, 1:2000, abcam, USA) overnight at 4°C. Next, the blots were incubated with goat anti-rabbit or anti-mouse, horseradish peroxidase conjugated secondary antibody and visualized by the enhanced chemiluminescence (ECL) detection, then exposed to X-ray films (Kadak). The protein bands were quantified by densitometry. The densitometry values for the proteins of interest were corrected for protein loading using GAPDH.

Statistical analysis

Statistical analyses were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA), and values expressed as mean values \pm standard deviation (SD).

Student's t-test was used to determine significant differences between groups. Data were considered statistically significant at P<0.05.

Results

BAG2, BAG5 expression was increased in MPTP-lesioned mice

Firstly, we need to confirm the association between BAG family protein and PD. We used the classical MPTP-lesioned mouse model of PD and checked whether the levels of BAG1, BAG2, BAG3, BAG5 protein changed in the SNpc. Morphological observations also showed a decrease of TH-immunoreactivity in SN sections. As expected, the number of TH-positive neurons significantly decreased after MPTP administration in MPTP group, to 38% of control group. Western blot analysis showed that compared with control group, BAG2 and BAG5



Figure 4. Effect of the rasagiline on the expression of TH, BAG2, BAG5 in SN shown by immunohistochemistry. A. MPTP-lesioned mice treated with rasagiline or water were transcardially perfused, then their brains were fixed for immunohistochemistry. Dopaminergic neurons, as identified by immunostaining against TH (red) expressed BAG2, BAG5 (red) (100×). B. The histogram represents the MPTP/Rasagiline group and MPTP/Water group. TH-positive, BAG2-positive, BAG5-positive cells were quantified across selected MPTP-sensitive sections. The neuroprotective effect of rasagiline was accompanied by further increase of BAG2, BAG5 protein. Data are expressed as the mean \pm SD (n=3). *P<0.05, compared with the MPTP/Water group.

protein levels were increased in MPTP group (P<0.05 for BAG2; P<0.05 for BAG5), while BAG1, BAG3 unchanged (**Figure 1**). Accordingly, immunohistochemistry found significantly in-

creased BAG2-positive ce-IIs and BAG5-positive cells (P<0.05 for BAG2; P<0.05 for BAG5) with reduction in THpositive cells in MPTP group (**Figure 2**). Increased BAG2, BAG5 level in MPTP mouse model was consistent with the previous observation that BAG5 was up-regulated in MPP⁺-treated PC12 cells.

Rasagiline treatment resulted in further increase of BAG2, BAG5 protein after intoxication with MPTP

Since we had got the result above, we sought to examine whether BAG proteins are involved in the neuroprotection by rasagiline. Rasagiline or water was given in MPTP/ Rasagiline group and MPTP/ Water group respectively after systemic administration of MPTP. We observed the neuroprotective effect of rasagiline in MPTP-Rasagiline group as shown by higher TH protein level and more TH-positive neurons by immunohistochemistry in SN sections (P<0.05) (Figures 3, 4). Compared to MPTP/Water group, protein levels of BAG2, BAG5 were furtherly increased in MPTP/Rasagiline group as demonstrated by western blotting (P<0.05 for BAG2; P<0.05 for BAG5) (Figure 3) and immunohistochemistry (P<0.05 for BAG2; P<0.05 for BAG5) (Figure 4). These findings showed that continuous administration of rasagiline after the MPTP treatment can further up-regulate BAG2, BAG5 protein levels and restore the reduction of TH immunoreactive cells in the SNpc.

Discussion

Previous studies have found both rasagiline and up-regulation of BAG5 can inhibits MPP⁺-

induced apoptosis through induction of antiapoptotic Bcl-2 and down-regulation of apoptogenic Bad, Bax [5, 6, 8, 11]. The current study for the first time examined the four BAG family protein changes in dopaminergic neurons of MPTP mouse model. Significant increase of BAG2, BAG5 levels were detected in MPTPlesioned mice (Figures 1, 2). Post-MPTP treatment with rasagiline markedly increased the number of dopaminergic neurons in the SN of the MPTP/Rasagiline group (Figure 4). Interestingly, this neuroprotective effect of rasagiline was accompanied by further increase of BAG2, BAG5 protein (Figure 3). The current finding that rasagiline treatment resulted in further increase of BAG2, BAG5 suggested the involvement and a positive role of BAG2, BAG5 in neuroprotection of rasagiline through apoptosisrelated proteins.

BAG proteins are characterized by a common conserved region located near the C terminus. termed the BAG domain (BD) that mediates direct interaction with the ATPase domain of Hsp70/Hsc70 molecular chaperone [9, 14]. Among them, BAG1 was better studied. BAG1 was found to have a regulatory effect of Hsp70 molecular chaperones [14]. BAG2 has been shown to interact with CHIP, Hsp70, MAPKAP through cochaperone-dependent regulatory mechanism [9, 10, 15]. The BAG2-Hsp70-CHIP complex has been shown to be part of a degradation pathway for tau, a protein implicated in Alzheimer's disease [16]. BAG5 is a unique member of the BAG family proteins in that it contains four BDs. Studies showed that BAG5 plays an important role in Parkinson disease through the interactions with Parkin E3 ligase, Hsp70 chaperone, CHIP [9, 10, 17, 18]. By modulating CHIP E3 ubiquitin ligase activity, BAG5 can mitigate CHIP-mediated production of toxic α-Syn oligomers [19].

Since BAG proteins are co-chaperones with complex networks, other pathways are worth to be explored such as the above mentioned UPS and autophagy protein degradation pathways.

In conclusion, we found up-regulated BAG2, BAG5 protein level against MPTP-induced neurotoxicity and the involvement of BAG2, BAG5 in neuroprotective effect of rasagiline. This preliminary finding implicates the underlying role of BAG protein in neurodegeneration and broadens the aspects of molecular mechanism on rasagiline neuroprotection.

Acknowledgements

This work was supported by by grant from the Major State Basic Research Development Program of China (973 Program) (2011CB510001), grant from the National Natural Science Foundation of China (30900469, 81171198, 8120-0870, 81000542, 81130021, 81371405, 81-361120404, 81571248, 81430023).

Disclosure of conflict of interest

None.

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