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Maneb and Mancozeb Increase Amyloid β Precursor Protein Expression and Activate PKR

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Abstract

Background: Oligomers of amyloid β (Aβ) in the brain correlate with synapse loss and dementia. The fungicide maneb (MB) and mancozeb (MZ) have been shown to activate the transcription factor NF-κB. Activation of NF-κB signaling pathway could initiate the transcription of β-amyloid precursor protein (AβPP) leading to increase Aβ level. Studies showed the cells treated with $Aβ_{42}$ have the elevated level of phosphorylated double-stranded RNA dependent protein kinase (PKR) which is found to deteriorate the neurons in AD brains.

Objective: To reveal the effects of MB and MZ on AβPP and Aβ42 expressions and to elucidate the role of PKR in response to MB and MZ.

Methods: Western blot analysis and ELISA assay were conducted to investigate the expressions of AβPP and $Aβ_{42}$ in PC12 cells treated with MB and MZ. The activation of PKR was determined by western blotting the level of phosphorylated PKR at Thr446 in SH-SY5Y cells treated with MB and MZ.

Results: MB and MZ increased AβPP and $Aβ_{42}$ expressions in a dose-dependent manner. MB and MZ transiently activated PKR.

Conclusion: Studies showed that MB and MZ enhance parkinsonian toxin MPP+ cytotoxicity and trigger DNA damage. The results from this study further revealed that MB and MZ are associated with the increase of AβPP and $Aβ_{42}$ expressions and elucidated that the PKR signaling pathway involves in MB and MZ induced cytotoxicity. This study provides the evidence about the relationship between fungicides and neurodegenerative diseases.

Keywords: Maneb; Mancozeb; AβPP; $Aβ_{42}$; PKR

Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases affecting older people. The etiology of AD is multifactorial including genetic and environmental factors. The association between the exposure to environmental toxins with the pathogenesis of AD has been suggested [1,2]. Tartaglione et al. [3] indicated that exposure to environmental chemicals in the early stage of life can interfere with developmental programming which increases the susceptibility to develop neurodegenerative diseases at a later life stage. The brains of AD patients present extracellular deposits mainly composed of a set of hydrophobic peptides called amyloid β-peptides (Aβ) [4,5]. Aβ is generated from β-amyloid precursor protein (AβPP) by secretase cleavages [6]. Some neurotoxins have been shown to elevate β-amyloid precursor protein (AβPP) expression, increasing β-amyloid (Aβ) peptide levels [7,8]. Oligomers of Aβ in the brain correlate with synapse loss and dementia [9]. Studies have also suggested that $Aβ_{42}$ is much more neurotoxic than $Aβ_{40}$ and is responsible for forming the plaques that are associated with Alzheimer's disease [10,11].

Increased evidences indicated that excessive or prolonged inflammatory reaction has a strong association with neurodegeneration [12,13]. The NF-κB (nuclear factor κB) transcription factor family which involves in cellular responses to various inflammatory stimuli is activated in AD brain [13,14]. NF-κB signaling pathway plays an unique role in neuronal survival and in increased vulnerability to neuronal cell death (13). Elevated activated NF-κB p65 increases Aβ production by enhancing endogenous beta site amyloid precursor protein cleaving enzyme-1 (BACE-1) transcription. Activation of NF-κB signaling pathway could also activate the transcription of AβPP [15]. The fungicide maneb (MB) and mancozeb (MZ) used in this study both contain manganese, which in high doses can be neurotoxic, and have been shown to activate the transcription factor NF-κB [16]. However, whether MB and MZ can increase AβPP and Aβ expressions is not clear.

Increased double-stranded RNA dependent protein kinase (PKR) expression is evidenced with aging and/or present in some neurodegenerative diseases [17-23]. Activated PKR is found to deteriorate the neurons in AD brains and also can regulate inflammatory responses of neurons [24]. PKR is one of several eukaryotic translation initiation factor 2α (eIF2α) kinases which are regulated by stresses. Phosphorylating eIF2α by eIF2α kinases will inhibit its function on protein translation. Studies showed that phosphorylation of PKR and eIF2α is increased in the cells treated with $Aβ_{42}$. The molecular connection between PKR and mTOR also has been demonstrated in response to $Aβ_{42}$ [21]. Dysregulation of PI3K/Akt/mTOR signaling pathway contributes to the pathogenesis of AD. Hugon et al. [23] indicated that PKR involves in Aβ synthesis via BACE 1. This study was also aimed to elucidate the involvement of PKR in response to MB and MZ in SHSY5Y cells.

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Materials and Methods

Cell culture

Human neuroblastoma (SHSY5Y) and rat pheochromocytoma (PC12) cell lines were obtained from American Type Culture Collection (ATCC Inc). Both cell lines have been used for Alzheimer studies. Briefly, PC12 cells were grown in fresh media containing Dulbecco's Modified Eagle's Medium (DMEM), with 10% fetal bovine serum (FBS), 5% heat-inactivated donor horse serum, 50 µg/ml gentamicin in a humidified atmosphere of 37°C and 5% CO_2. SHSY5Y cells were grown in fresh media containing DMEM/F12, 10% FBS and 50 µg/ml gentamicin in a humidified atmosphere of 37°C and 5% CO_2. Cells were set up on a day before the chemical treatments to ensure that the cell density on the day of experiment was about 80% confluence.

Chemical treatment

Manganese-containing dithiocarbamates, maneb (MB) and mancozeb (MZ), were obtained from Sigma Aldrich. The various experimental concentrations of MB and MZ were freshly prepared 15 minutes prior to treatment. First, MB and MZ were dissolved in dimethylsulfoxide (DMSO) and then diluted to the experimental concentrations by 1x phosphate-buffered saline (PBS). The final DMSO concentration in the cells was less than 0.1%, which is below toxic dose. These tested concentrations of MB and MZ were chosen based on previous studies [16,24]. Cells were treated with their indicated chemicals and the control cells was treated with PBS for the indicated time periods at 37°C and 5% CO_2 incubator.

Western blot analysis

After chemical exposures, cells were lysed with a mixture of Mammalian protein extraction reagent (M-PER) and Halt™ protease/phosphatase inhibitor cocktail (Thermo Fisher). The concentration of protein samples was determined by using Bio-Rad DC (detergent compatible) protein assay reagents (Bio-Rad, Hercules, CA).

Thirty (30) µg of protein samples from each treatment condition were resolved via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked in PBS/0.05% Tween 20, containing 5% FBS, and were then probed overnight with primary antibody specific for AβPP (Sigma, A8717), $Aβ_{42}$ (Sigma, A1650), β-actin (Sigma, A2228), PKR (Santa Cruz, SC707), p446 PKR (Santa Cruz, SC16565) at concentration of 1 µg/µl. The antibody was detected with corresponding horseradish peroxidase-linked secondary antibody. Blots were developed using Super Signal West Pico Chemiluminescent Substrate detection reagents from Pierce. Membranes were then stripped with Stripping buffer for 15 min at room temperature and re-probed with loading control antibody. Chemilluminescent signals were captured using Geliance 600 imaging system (Perkin Elmer, Shelton, CT) and analyzed by GeneTools software (Syngene, Frederick, MD).

ELISA for quantifying Aβ42

The level of $Aβ_{42}$ was detected with Amyloid beta 42 ELISA Kit from Invitrogen following the manufacturer's protocol. Briefly, the samples were added onto a 96-well plate, which is coated with a monoclonal antibody to N-terminus of $Aβ_{42}$. The samples containing $Aβ_{42}$ antigen were captured to the immobilized antibody coated on a 96-well plate. After washing, a rabbit monoclonal antibody specific for the C-terminus of $Aβ_{42}$ was added. The bound rabbit antibody was then detected by the use of a horseradish peroxidase (HRP)-labeled anti-rabbit antibody. After washing, a substrate solution was added, which is acted upon by the bound HRP enzyme to produce a signal. The intensity of the signal is directly proportional to the concentration of $Aβ_{42}$ in the samples.

Statistics

All experiments were performed at least in triplicate, and results are reported as means ± SEM. Statistical significance was determined using one way analysis of variance (ANOVA) followed by Dunnett's post-hoc test ($p<0.05$, with control at 100%) by using GraphPad PRISM® 6 software.

Results and Discussion

MB and MZ increase AβPP and Aβ42 oligomer expressions in PC12 cells

Alzheimer's disease (AD), like many other neurodegenerative diseases, has a complex mechanism and is not fully understood. The major component of amyloid plaques found in AD patients' brains is amyloid β-peptides (Aβ) which is the cleavage product of β-amyloid precursor protein (AβPP). Elevating AβPP expression and Aβ peptide levels can been triggered by neurotoxins, such as diethyldithiocarbamate [1,8]. Maneb (MB) and mancozeb (MZ) which are also dithiocarbamate compounds have been shown to cause DNA damage and enhance parkinsonian toxin MPP^+ toxicity [16,25]. Manganese which MB and MZ both contain is known neurotoxic in high doses [26]. Tong et al. [27] indicated that excessive Mn exposure increases Aβ by inhibiting its degradation. However, the association of MB and MZ with AD is not so clear.

In order to determine whether MB and MZ can increase AβPP and $Aβ_{42}$ expressions, PC12 cells were treated with MB and MZ at different concentrations (5-20 µM) for 24 hours and the protein samples were then analyzed by western blot analysis. The western blot results showed that cells treated with MB for 24 hours have significantly increased AβPP expression by 133% in the cells treated with 20 µM MB (Figure 1). The cells treated with MZ for 24 hours have significantly elevated AβPP expression levels by 39% in the cells treated with 5 µM MZ and 50% in the cells treated with 20 µM MZ (Figure 2).

The expression of $Aβ_{42}$ was also monitored by western blot analysis. The $Aβ_{42}$ expression detected in this experiment was of an oligomer.

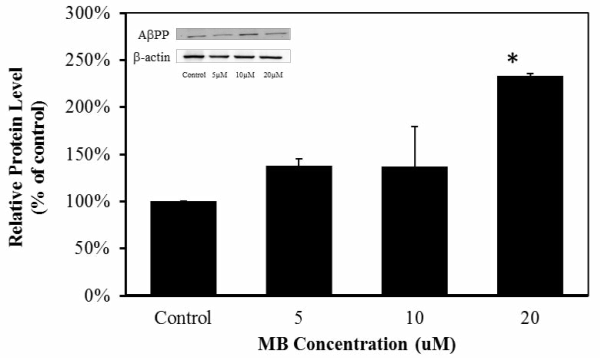

Figure 1: Effect of maneb (MB) on AβPP expression in PC12 cells. The relative levels of AβPP in PC12 cells treated with MB for 24 hours were determined by western blot analysis. Beta actin expression was also measured as the loading control. The integrated density values (IDV) of western blot images of AβPP were normalized with the IDV of the corresponding β-actin images. The final data for all chemically treated groups were expressed as a percentage of the control data. The data of control samples were taken as 100%. * vs. control with $p<0.01$.

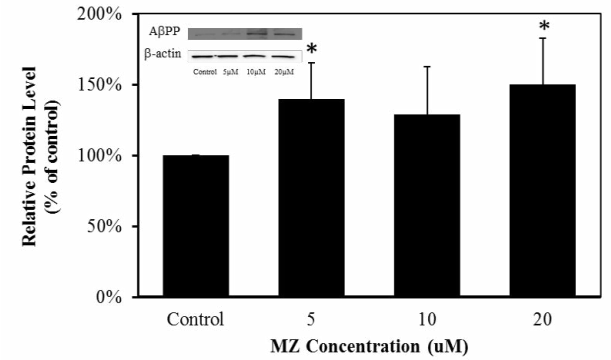

Figure 2: Effect of mancozeb (MZ) on AβPP expression in PC12 cells. The relative levels of AβPP in PC12 cells treated with MZ for 24 hours were determined by western blot analysis. Beta actin expression was also measured as the loading control. The integrated density values (IDV) of western blot images of AβPP were normalized with the IDV of the corresponding β-actin images. The final data for all chemically treated groups were expressed as a percentage of the control data. The data of control samples were taken as 100%. * vs. control with p<0.05.

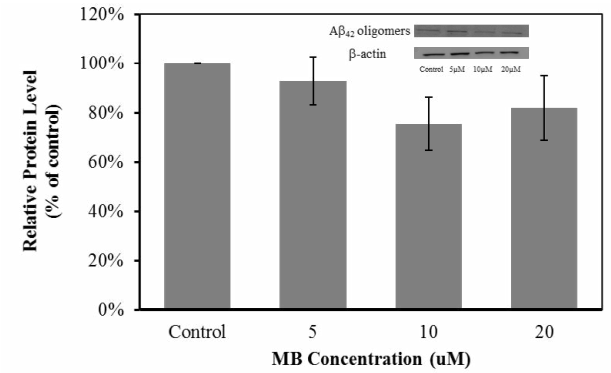

Figure 3: Effect of maneb (MB) on Aβ42 oligomer expression in PC12 cells. The relative levels of Aβ42 oligomer in PC12 cells treated with MB for 24 hours were determined by western blot analysis. Beta actin expression was also measured as the loading control. The integrated density values (IDV) of western blot images of Aβ42 oligomer were normalized with the IDV of the corresponding β-actin images. The final data for all chemically treated groups were expressed as a percentage of the control data. The data of control samples were taken as 100%.

The results showed that cells treated with MB for 24 hours did not have the significant changes in Aβ42 oligomer expression (Figure 3). But cells treated with MZ for 24 hours have significantly increased $A\beta_{42}$ oligomer expression levels, 55% increase in the cells treated with 10 μM MZ and 63% increase in the cells treated with 20 μM MZ (Figure 4).

These results showed that MB and MZ enhanced AβPP expression in a dose-dependent manner (Figures 1 and 2). Interestingly, the increased level of $A\beta_{42}$ oligomers was only observed in cells treated with MZ in a dose-dependent manner (Figure 4). The level of $A\beta_{42}$ oligomers in cells treated with MB did not change in a dose-dependent manner (Figure 3). Since Aβ is the cleavage product of AβPP, the smaller increases of AβPP expression in the cells treated with MZ might be due to the cleavage of AβPP. Therefore, the cells treated with MZ have the higher increases of the $A\beta_{42}$ oligomers levels. On the other hand, the level of $A\beta_{42}$ oligomers in MB treated cells might be increased after longer chemical exposure.

MB and MZ increase Aβ42 monomer expression in PC12 cells

To further investigate whether MB and MZ can increase Aβ42 monomer expression, PC12 cells were treated with MB and MZ at different concentrations (5-50 μM) for 24 hours. The level of Aβ42 monomer expression was determined through ELISA analysis. The results showed that cells treated with MB for 24 hours increased Aβ42 monomer expression from 1.4 ng/mg protein (cells treated with 5 μM) to 3.4 ng/mg protein (cells treated with 50 μM) (Figure 5). When cells were treated with MZ for 24 hours, Aβ42 monomer expression increased from 3.5 ng/mg protein (cells treated with 5 μM) to 22.9 ng/mg protein (cells treated with 50 μM) (Figure 6).

ELISA assay results from this study showed the increases of Aβ42 monomer expressions in cells treated with MB and MZ in a dose-dependent manner (Figure 5 and 6). MB triggered less of an increase in Aβ42 monomer expression as compared with MZ. Studies indicated that Aβ oligomers are neurotoxic and can impair synaptic plasticity and Aβ monomer has neuroprotective properties [28-32]. These results along with previous cytotoxicity results of MB and MZ, reinforce that MZ is much more toxic compared to MB [16,26,33]. These results also were consonant with western blot analysis data of Aβ42 oligomer

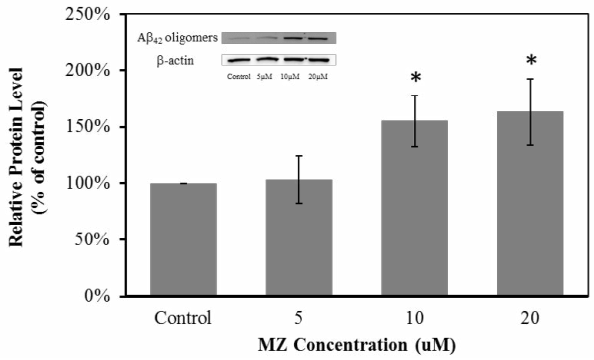

Figure 4: Effect of mancozeb (MZ) on Aβ42 oligomer expression in PC12 cells. The relative levels of Aβ42 oligomer in PC12 cells treated with MZ for 24 hours were determined by western blot analysis. Beta actin expression was also measured as the loading control. The integrated density values (IDV) of western blot images of Aβ42 oligomer were normalized with the IDV of the corresponding -actin images. The final data for all chemically treated groups were expressed as a percentage of the control data. The data of control samples were taken as 100%. * vs. control with p<0.05.

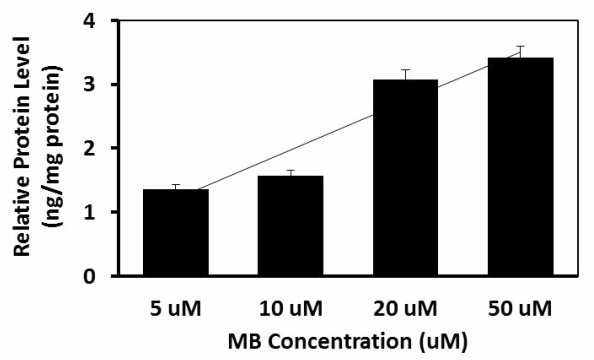

Figure 5: Effect of maneb (MB) on Aβ42 monomer expression in PC12 cells. The relative levels of Aβ42 monomer in PC12 cells treated with MB for 24 hours were determined by ELISA. Protein concentration was measured to normalize ELISA data. The final data for all chemically treated groups were expressed as ng of Aβ42 monomer per mg of protein.

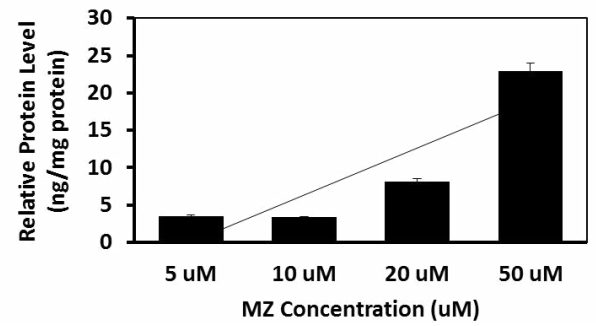

Figure 6: Effect of mancozeb (MZ) on Aβ42 monomer expression in PC12 cells. The relative levels of Aβ42 monomer in PC12 cells treated with MZ for 24 hours were determined by ELISA. Protein concentration was measured to normalize ELISA data. The final data for all chemically treated groups were expressed as ng of Aβ42 monomer per mg of protein.

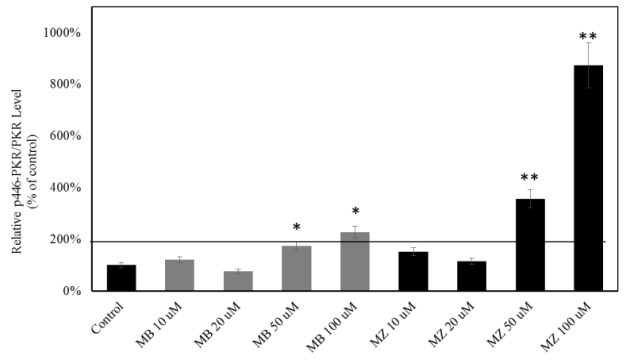

Figure 7: Effects of MB and MZ on PKR activation in SH-SY5Y cells. The relative levels of phosphorylated PKR at Thr446 (p446-PKR) in SH-SY5Y cells treated with MB and MZ for 8 hours were determined by western blot analysis. Total PKR expression was also measured as the loading control. The integrated density values (IDV) of p446-PKR western blot images were normalized with the IDV of the corresponding PKR images. The final data for all chemically treated groups were expressed as a percentage of the control data. The data of control samples were taken as 100%. * vs. control with p<0.05; ** vs. control with p<0.01.

expressions that MZ triggered larger increases in Aβ42 oligomer expressions as compared with MB.

MB and MZ transiently activate PKR in SH-SY5Y cells

PKR constitutively expressed in mammalian cells is one of serine-threonine kinases and can phosphorylate eIF2α to block protein synthesis leading to cell death in response to cellular stresses. Numerous studies suggested that PKR is dysregulated in cellular and animal models of neurodegenerations and in the brains of AD patients [21-23,34,35]. Morel et al. [21,22] suggested that PKR activation could be an early indicator of the neuronal death in AD.

Studies suggested that PKR signaling pathway is activated in response to $A\beta_{42}$. To determine the involvement of PKR in response to MB and MZ, SH-SY5Y cells were treated with MB and MZ at 10-100 μM for 8 and 16 hours and the protein samples were then analyzed by western blot analysis. The results showed that 8 hours of MB and MZ exposures increased the level of phosphorylated PKR at T446 (p446 PKR) in a dose-dependent manner (Figure 7). MZ triggered stronger PKR activation as compared to MB.

The level of phosphorylated PKR (p446 PKR) still increased after 16 hours of MB and MZ exposures (Figure 8). For cells treated with 10 and 20 μM of MB for 16 hours, the increases were higher as compared with cells treated with 10 and 20 μM of MB for 8 hours. The increases after 16 hours of 50 and 100 μM MZ exposures were much less compared to those treated for 8 hours. This indicated that the activation of PKR was transient.

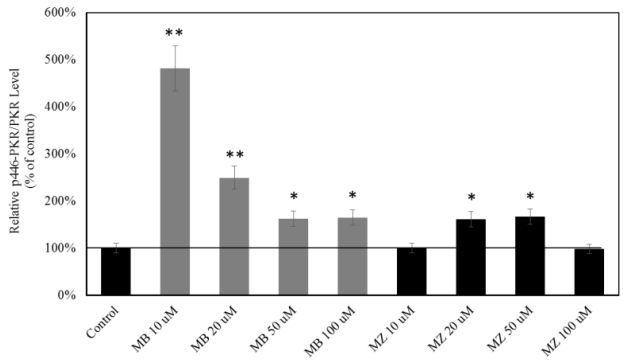

Figure 8: Effects of MB and MZ on PKR activation in SH-SY5Y cells. The relative levels of phosphorylated PKR at Thr446 (p446-PKR) in SH-SY5Y cells treated with MB and MZ for 16 hours were determined by western blot analysis. Total PKR expression was also measured as the loading control. The integrated density values (IDV) of p446-PKR western blot images were normalized with the IDV of the corresponding PKR images. The final data for all chemically treated groups were expressed as a percentage of the control data. The data of control samples were taken as 100%. * vs. control with p<0.05; ** vs. control with p<0.01.

Marchal et al. indicated that PKR is able to phosphorylate p53 tumor suppressor, which activates RTP801 [36]. RTP801 activation can hinder mTOR activity leading to cell death [36]. In 2014, Cheng et al. revealed that MB and MZ can up-regulate RTP801 [26]. This study further elucidated that PKR could be the up-stream signaling pathway of RTP801 in response to MB and MZ.

Conclusion

Studies showed that MB and MZ enhance MPP^+ cytotoxicity and trigger DNA damage. RTP801 activation via NF-κB in response to MB and MZ was evidenced. The results from this study further revealed that MB and MZ were associated with the increases of AβPP and $A\beta_{42}$ expressions and elucidated that the PKR signaling pathway involved in MB and MZ induced cytotoxicity. The activated PKR could lead to the activation of RTP801 and cell death. This study provides the evidence about the relationship between fungicides and neurodegenerative diseases.

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