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Title: Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury

Article Type: Research Paper

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Abstract: Methylmercury (MeHg) is well-known for causing irreversible damage in the central nervous system as well as a risk factor for inducing neuronal degeneration. However, the molecular mechanisms of MeHg-induced neurotoxicity remain unclear. Here, we investigated the effects and possible mechanisms of MeHg in the mouse cerebrum (in vivo) and in cultured Neuro-2a cells (in vitro). In vivo study showed that the levels of LPO in the plasma and cerebral cortex significantly increased after administration with MeHg (50 μg/kg/day) for 7 consecutive weeks. MeHg could also decrease glutathione level and increase the expressions of caspase-3, -7, and -9, which were accompanied with Bcl-2 down-regulation and up-regulation of Bax, Bak, and P53. Moreover, treatment of Neuro-2a cells with MeHg significantly reduced cell viability, increased oxidative stress damages, and induced several features of mitochondria-dependent apoptotic signals, including increased sub-G1 hypodiploids, mitochondrial dysfunctions, and the activations of PARP, and caspase cascades. These MeHg-induced apoptotic-related signals could be remarkably reversed by antioxidant NAC. MeHg also increased the phosphorylation of ERK1/2 and p38, but not JNK. Pharmacological inhibitors NAC, PD98059, and SB203580 attenuated MeHg-induced cytotoxicity, ERK1/2 and p38 activation, MMP loss, and caspase-3 activation in Neuro-2a cells. Taken together, these results suggest that the signals of ROS-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic pathways that are involved in MeHg-induced neurotoxicity.

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Declarations

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Please state any sources of funding for your research

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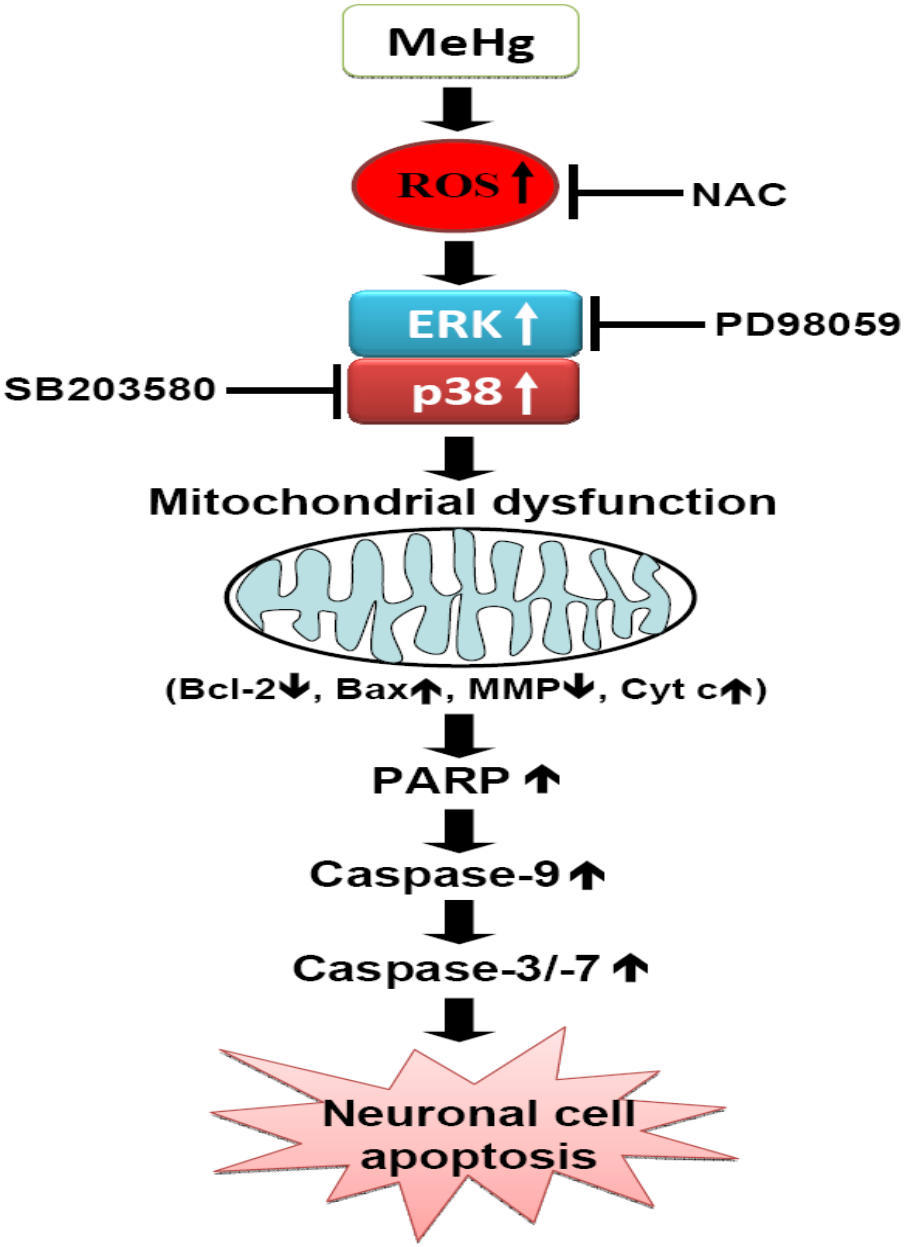
Graphical Abstract



Professor James P. Kehrer
Editor-in- Chief
Toxicology Letters Editorial Office

Dear Professor:

The manuscript (TOXLET-D-11-00006) entitled "**Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury**" was carefully revised according to your and reviewers` valuable suggestions. Please reconsider to publish this revised manuscript in Toxicology Letters. Further suggestions on our revised manuscript will be greatly appreciated.

With the best wishes,

Sincerely yours,

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Reply to editor's comments:

Q1. Reviewer 1's comments were relatively minor. However, Reviewer 2 indicated a need for addidtional quantitative data to suppor the qualitative data presented. As Editor, I would ask that you shorten the abstract. In addition, the overall paper is too long and must be shortened by 20%.

Ans. We have been reduced in length and re-organized our revised manuscript. The errors of English grammar have also been carefully corrected and proofread in this manuscript by a native English-speaking colleague. If further editorial corrections are needed, we will be pleased to revise according to your suggestions.

Reply to reviewer' 1 comments:

Q1. The results should be presented as mean with standard deviations, standard errors are not correct to show for biological experiments.

Ans. We have been re-calculated the results and presented as mean ± standard deviations (S.D.) (please see P13, L23-P14, L4, and in the '**Figures**' section of revised manuscript).

Q2. The introduction/discussion should be improved with details on: (1) was is new in this study?; (2) environmental relevance of the concentrations/doses used, uptake/efflux of methylmercury in vitro and in vivo.

Ans. We have been improved and added with the detailed descriptions in our manuscript according to Reviewer's suggestion, as follows:

(1). Although there is many studies have indicated about MeHg-induced toxicological effects, the signaling mechanisms of MeHg-induced neurotoxic effects are not yet understood. In this study, we attempted to explore the role of oxidative stress-mediated signaling in neuronal cell death by investigating whether ROS generation, mitochondrial dysfunction, caspase cascades, and/or ERK/p38-MAPK activation are involved in MeHg-induced apoptosis in Neuro-2a cells. The present *in vivo* and *in vitro* results provides evidence that MeHg is capable of inducing neuronal degeneration and apoptosis, and more importantly, reveals the molecular basis for its effects. This study has clearly demonstrated that MeHg-induced oxidative stress causes apoptosis in neuronal cells through ERK1/2- and p38-MAPK activation regulated mitochondrial dysfunction triggered PARP and caspase-9 activation, and involved in caspase-3/-7-mediated mechanism (P6, L22-25 and P23, L2-8 of the

revised manuscript).

(2). ‘Exposure to MeHg (1-10 μM) has reported to induce toxic effects by production of oxidative stress, which alters cellular function and eventually results in pathophysiological injury and cell death in various cells, including neuronal cells’ in the ‘**1. Introduction**’ section (P5, L19-21);

‘Many studies have indicated that MeHg is a potent neurotoxicant affecting both the developing and mature central nervous system, and can cause severe neuropathophysiological disorders with exposure to high concentrations (0.5-40 ppm in drinking water or 0.2-2 mg/kg/day, for more than 7 consecutive days) *in vivo* system, including loss of neurons in the calcarine cortex, cerebellar Purkinje, and granule cells, leading to vision, motion, or postural abnormalities (Carvalho et al., 2007; Chuu et al., 2007; Dare et al., 2003; Goulet et al., 2003; Onishchenko et al., 2007). Exposure to MeHg (20-50 μg/kg) in mice, which was the possible dosage from food ingested in MeHg-contaminated areas (8.03-174 μg-MeHg/kg, even more than 200 μg-MeHg/kg), caused severe neurotoxic injuries has also reported, that accompanied with significant mercury accumulation (113.0-241.8 ng-mercury/g wet. wt.) and oxidative stress generation in the brain regains (Grandjean et al., 1992; Huang et al., 2008 and 2011; Maramba et al., 2006; Qiu et al., 2008).’ In the ‘**4. Discussion**’ section (P19, L1-18).

Q3. The study used relative high concentrations of MeHg, low concentrations are represented by picomolar range to low nanomolar concentrations, therefore the text should be modified accordingly.

Ans. We have been corrected this misunderstanding in the revised text.

Q4. Results: the use of NAC did not fully reverse the effect of MeHg therefore the authors should discuss more in details the occurrence of other mechanisms of MeHg toxicity.

Ans. We have discussed more in details the occurrence of possible mechanisms of MeHg-induced neurotoxicity, as follows:

‘The antioxidant NAC could effectively prevent, but not fully reverse, these MeHg-induced responses. These findings indicate that oxidative stress is caused by MeHg exposure, and it may be involved in MeHg-induced neuronal degeneration and cell apoptosis. Furthermore, Fujimura et al (2009) has reported that the expression of Rac1 (Rho-family protein) can be down-regulated and ultimately led to apoptosis in MeHg-exposed neuronal cells. Tofighi et al (2011) has also indicated that MeHg induces caspase-independent cell death via parallel activation of calpains and lysosomal proteases in hippocampal neurons. However, the critical roles of these signals in MeHg-induced neurotoxicity will still investigation in the future.’ (P20, L13-21 of the revised manuscript).

Q5. Overall, a nice and complete study.

Ans. My colleagues and I thank a lot for the review’s commendation.

Reply to reviewer' 2 comments:

Q1. This study examined the effects of low dose methylmercury exposure in adult male mice in vivo and in cell culture using neuro-2a cells. A number of different parameters were investigated including cell viability, caspase expression, ROS formation, intracellular glutathione and others. While neuroblastoma cell lines are often used to test for neurotoxic properties of various compounds, it must be kept in mind that transformed cell lines do not always display morphological and biochemical characteristics that are identical to the originating cell type. (see Keith T. LePage et al., On the Use of Neuro-2a Neuroblastoma Cells Versus Intact Neurons in Primary Culture for Neurotoxicity Studies. 2005: 17(1) Pages 27-50.) This means that neuroblastoma cells may not always respond in the same way as neurons to toxin exposure. One always needs to exercise some degree of caution when interpreting cytotoxicity data derived from the use of neuroblastoma cell lines. While this study presents interesting data, the authors do not discuss the possible differences that might be present between neuroblastoma and neuronal responses.

Ans.

1. Murine neuroblastoma cell line: Neuro-2a (CCL-131, American Type Culture Collection, Manassas, VA, USA) is established by R. J. Klebe and F. H. Ruddle from a strain A albino mouse (Klebel and Ruddle, 1969), and often used to test or explore the mechanisms for neurotoxic properties of various compounds (Dickey et al., 2010; Gross et al., 2010; Hsieh et al., 2011). Despite some limitations about neuroblastoma cell lines used in neurotoxicity studies maybe consider, including: (1). transformed cell lines do not always display morphological and biochemical characteristics that are identical to the originating cell type; (2). LePage et al. (2005) has indicated that it needs to exercise caution in interpreting negative

cytotoxicity data derived from the use of neuroblastoma cell lines (the responses of cell lines to toxin exposure may differ and lower sensitivity from that of primary cerebral granule neurons), these cells are still the useful biological material to apply the neurotoxicological effect studies *in vitro* and there are many studies choosing the neuro-2a cells to examine the cytotoxic effects of toxic metals (Abreo et al., 1999; Johnson et al., 2005; Wang et al., 2010; Yin et al., 2005). Therefore, our study has chosen the neuro-2a cells to investigate the toxicological effects and possible mechanisms involved in MeHg-induced neurotoxicity.

2. Moreover, in order to keep the best morphological and biological characteristics of neuro-2a cells in our studies, all assays were conducted within 10-15 cell passages after unfreezing the cells from the liquid nitrogen (this sentence has been clearly described in P8, L7-9 of the '*2.1 Cell Culture*' Section).
3. It may be different responses between the primary cerebral granule neurons and the neuroblastoma cell lines to same toxin exposure. Thus, the differently neurotoxic responses of MeHg-induced between the primary cerebral granule neurons and the Neuro-2a cells will need to explore, in the future.

Reference:

Abreo, K., Abreo, F., Sella, M.L., Jain, S., 1999. Aluminum enhances iron uptake and expression of neurofibrillary tangle protein in neuroblastoma cells. J. Neurochem. 72, 2059-64.

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cytotoxic stereoisomers of malyngamide C, 8-epi-malyngamide C and 8-O-acetyl-8-epi-malyngamide C, from the marine cyanobacterium Lyngbya majuscula. Phytochemistry 71, 1729-35.

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Johnson, V.J., Kim, S.H., Sharma, R.P., 2005. Aluminum-maltolate induces apoptosis and necrosis in neuro-2a cells: potential role for p53 signaling. Toxicol. Sci. 83, 329-39.

Klebe, R.J., Ruddle, F.H., 1969. Neuroblastoma: Cell culture analysis of a differentiating stem cell system. J. Cell Biol. 43, 69A.

LePage, K.T., Dickey, R.W., Gerwick, W.H., Jester, E.L., Murray, T.F., 2005. On the use of neuro-2a neuroblastoma cells versus intact neurons in primary culture for neurotoxicity studies. Crit. Rev. Neurobiol. 17, 27-50.

Wang, X., Meng, D., Chang, Q., Pan, J., Zhang, Z., Chen, G., Ke, Z., Luo, J., Shi, X., 2010. Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway. Environ. Health Perspect. 118, 627-34.

Yin, H., Too, H.P., Chow, G.M., 2005. The effects of particle size and surface coating on the cytotoxicity of nickel ferrite. Biomaterials 26, 5818-26.

Q2. The English grammar is generally adequate in this manuscript, but there still are quite a few minor corrections that need to be made throughout the text. These are cases of use of singular versus plural, present versus past tense, etc., that can easily be corrected. I have not provided a list of specific examples but consultation with a native English-speaking colleague whould be useful in this regard, in order to correct

these minor grammatic errors.

Ans. We have been carefully corrected and checked the English grammars and syntax mistake in this revised manuscript. If further editorial corrections are needed, we are being pleased to revise according your suggestions.

In the material and methods section:

Q1: the authors do not always provided complete information for the companies that were used to obtain supplies and/or equipment. Complete information needs to be provided the first time each company is mentioned in the text. Some references to companies are complete but some are lacking necessary information.

Ans: The companies' information has been clearly provided in the '**2. Materials and Methods**' section of revised manuscript.

Q2. In section 2.2 the authors need to state how long the cells were cultured in the 96 well plates.

Ans. We have been clearly described the sentence, as follows:

'Neuro-2a cells were seeded at 2×10^4 cells/well in 96-well plates and allowed to adhere and recover overnight. The cells were changed to fresh media and then incubated with MeHg (1-5 μM; Sigma-Aldrich, St. Louis, MO, USA) in the absence or presence of NAC (1 mM) or specific MAPK inhibitors (20 μM, Sigma-Aldrich) for 24 h.' (please see P8, L12-16 of the revised manuscript).

Q3. In section 2.4 it is not clear how long the methylmercury exposure occurred.

Ans. We have been clearly described the methylmercury exposure occurred in section 2.4, as follows: ‘Neuro-2a cells were seeded at 2×10^5 cells/well in a 24-well plate and allowed to adhere and recover overnight. The cells were changed to fresh media and incubated with MeHg in the absence or presence of NAC (1 mM, Sigma-Aldrich) for 24 h.’ (P9, L8-10 of the revised manuscript).

Q4. It also is not clear in several of the experiments that used cell lysates, how the authors were able to normalize to cell number between different groups when the cells were lysed.

Ans. We have been clearly described this part in the revised manuscript, as follows: ‘Protein levels of cell lysate samples were determined using the bicinchoninic acid protein assay kit with an absorption band of 570 nm (Pierce; Rockford, IL, UAS) to normalize the cell numbers between control and different MeHg-treated groups.’ (Please see: P10, L16-19 and P12, L9-10 of the revised manuscript).

Q5. In section 2.9 the authors should provide a citation that describes the guidelines that are provided by the Animal Research Committee of the China Medical University.

Ans. We have been provided the citations about described the guidelines that are provided by the Animal Research Committee of the China Medical University, as follows: ‘All experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC), and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of China Medical University

(http://cmurdc.cmu.edu.tw/LA/index.php).' (in P11, L9-13 of the revised manuscript).

Q6. At the end of section 2.9 the authors state that more than 12 mice were used in each in vivo group. The range of animal numbers should be provided.

Ans. The range of animal numbers of each group in this study were 12~15 mice (*n* = 12~15), and have been added this description in section 2.9 (P11, L17) and in '**Figure Legends**' section of the revised manuscript.

Q7. It is not clear what the authors mean by collecting blood "from the peripheral vessels". It is implied that the mice were decapitated and then trunk blood was collected from the severed neck, but that is not what is stated in the manuscript. The procedure for collecting blood needs to be modified.

Ans. We have been clearly described the procedure for collecting blood, as follows: 'All experimental mice were deep anesthesia by an intraperitoneal injection of pentobarbital (80 mg/kg) and the whole blood samples were collected from an eyehole vessel. Whole blood sample were centrifuged at 3,000 × *g* for 10 min, and plasma was obtained, and LPO levels was assayed immediately. At the same time, mice were sacrificed by decapitation under pentobarbital anesthesia, and the cerebral cortex were quickly removed and stored at liquid nitrogen until use, and then was analysis of LPO levels, GSH content, and apoptosis-related genes expression.' (in P11, L17-24 of the revised manuscript).

Q8. In section 2.12 it would be helpful if the authors would put the primers that were used in a table or chart instead of just listing them in the text. It is difficult to

understand the primers as they are listed in the text, so including a table or chart would be better.

Ans. All the PCR primers of this study used have been put in the '**Table 1.** Primer sequences used for the real-time quantitative RT-PCR analysis' (in P36 of the revised manuscript).

Q9. The last sentence in section 2.12 is not clear with respect to out the fold changes were calculated. This information needs to be described in more detail.

Ans. The statement of section in '2.12. *Real-time quantitative reverse-transcribed polymerase chain reaction (RT-PCR) analysis*', which about calculated data with respect to out the fold changes, has been clearly described, as follows:

'Data analysis was performed using StepOne^{TM} software (Version 2.1, Applied Biosystems). All amplification curves were analyzed with a normalized reporter (R_n: the ratio of the fluorescence emission intensity to the fluorescence signal of the passive reference dye) threshold of 0.2 to obtain the C_T values (threshold cycle). The reference control genes were measured with four replicates in each PCR run, and their average C_T was used for relative quantification analyses (the relative quantification method utilizing real-time PCR efficiencies (Pfaffl et al., 2002)). TF expression data were normalized by subtracting the mean of reference gene C_T value from their C_T value (ΔC_T). The Fold Change value was calculated using the expression $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ represents $\Delta C_{T\text{-condition of interest}} - \Delta C_{T\text{-control}}$. Prior to conducting statistical analyses, the fold change from the mean of the control group was calculated for each individual sample.' (in P13, L10-21 of the revised manuscript).

Several points need to be addressed in the results section:

Q1: First of all, in section 3.2, there is a reference at the bottom of the page to Figure 3. Do the authors mean to refer to Figure 3E? Otherwise, there is no specific reference to Figure 3E. In section 3.3 in line 6 from the bottom, the authors refer to Figures 3A and B right after Figure 5B and before Figure 5C. Do the authors mean to refer to Figures 5A and B?

Ans. We have been clearly described these points in the results section of revised manuscript, as follows:

(A). 'Figure 3' to 'Figures 3B, 3C, 3D, and 3E' (P16, L17);

(B). 'Figures 3A and B' to 'Figures 5A and B' (P17, L20).

Q2. A more general point that needs to be addressed is whether there is a real dose response seen in some of the experiments that are included in this study. Some data do appear to be showing a dose response. However, no data analysis has been provided to clearly demonstrate dose responses in any of the graphs that supposedly show a dose response. For example, Figure 3A is described as showing a dose response; as dose increased, cell viability decreased. The authors do not provide any statistical data to support this observation although they could easily do so.

Ans. Thanks a lot for your suggestion. In this study, we have been re-calculated data (presented as means ± standard deviations (S.D.)) and statistic analysis. Our statistical data did not support the observation about 'in a dose-dependent (response) or time-dependent manner' in MeHg-induced toxic effects. Thus, these misunderstands have been deleted and corrected in the results section of revised manuscript.

Q3. It also is the case for some of the experiments that the authors show Western blots but no quantitative data. It is stated in the figure legends that a minimum of 3 independent experiments were carried out, but no quantitative data are provided, only photomicrographs of a single representative blot. Quantitative data to support the observations should be provided for figures 5d, 6A, 6Bb, 6Bc, and supplementary figure 2.

Ans. We have been calculated and compiled statistic the quantitative data of Western blot for Figure 5D, 6A, 6Bb, 6Bc, and supplementary Figure 2 (please see the 'Figures' section and 'Supplementary Figures' section of revised manuscript).

In the Discussion section:

Q1. It is not clear in the discussion why the authors bring up Alzheimer's disease in the discussion in context of methylmercury exposure. The possible connection needs to be strengthened or removed from the discussion. The same point can be made for the last sentence in the conclusion although the authors mention neurodegenerative diseases in general in the conclusion and not Alzheimer's disease specifically in the last sentence.

Ans. We have been removed the part of referring to Alzheimer's disease in the discussion section and re-written the part 1 of 'Discussion' section (please see P19, L14-26 of the revised manuscript).

Q2. The authors mention "low dose" human exposure but what does that actually mean? There also is no clear discussion of what levels of methylmercury can be expected or observed for current human exposure and how those relate to doses used

in this study.

Ans. In this study, the mention ‘explored whether exposure to low dose of MeHg (50 μg/kg), which mimicked the human possible exposed dose in MeHg-contaminated areas...’ was according to epidemiological researches, which reported that MeHg level of foods in MeHg-contaminated areas is 8.03-174 μg-MeHg/kg, even more than 200 μg-MeHg/kg in some areas (Grandjean et al., 1992; Maramba et al., 2006; Qiu et al., 2008). The dosage (50 μg/kg MeHg) used in this study was lower as compared with the previous studies, which were exposed to high concentrations (0.5-40 ppm in drinking water or 0.2-2 mg/kg/day, for more than 7 consecutive days) *in vivo* system (Carvalho et al., 2007; Chuu et al., 2007; Dare et al., 2003; Goulet et al., 2003; Onishchenko et al., 2007). However, ‘Low dose’ or ‘Low concentration’ is presented by pico-molar/gram range to nano-molar/gram concentration. Thus, we have been corrected this misunderstanding and clearly described this part in the ‘4. Discussion’ section of revised manuscript (please see P19, L2-18 of the revised manuscript).

References:

Carvalho, M.C., Franco, J.L., Ghizoni, H., Kobus, K., Nazari, E.M., Rocha, J.B., Nogueira, C.W., Dafre, A.L., Muller, Y.M., Farina, M., 2007. Effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) on methylmercury-induced locomotor deficits and cerebellar toxicity in mice. Toxicology 239, 195-203.

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Onishchenko, N., Tamm, C., Vahter, M., Hokfelt, T., Johnson, J.A., Johnson, D.A., Ceccatelli, S., 2007. Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicol. Sci. 97, 428-37.

Qiu, G., Feng, X., Li, P., Wang, S., Li, G., Shang, L., Fu, X., 2008. Methylmercury accumulation in rice (Oryza sativa L.) grown at abandoned mercury mines in Guizhou, China. J. Agric. Food Chem. 56, 2465-8.

Specific points

Q1. The first "sentence" in section 3.2 is not a complete sentence as it is written: "whether the mechanisms involved in Me-Hg-induced neurotoxicity." is a phrase and not a complete sentence.

Ans. We have corrected this sentence in section 3.2 (P16, L1 of the revised manuscript).

Q2. In the middle of the second page of the discussion there is sentence that reads: Moreover, i also significantly decreased. There is no indication as to what "i" refers.

Ans. This mistake ‘Moreover, i also significantly decreased.’ has been corrected to ‘Moreover, MeHg (1-5 μM) exposure significantly decreased...’ in P20, L10 of the revised manuscript.

Q3. In the conclusion the authors refer to figure 9, but there is no figure 9. It would appear that the authors may mean to refer to figure 7.

Ans. It has been corrected in the ‘5. Conclusion’ section (in P23, L8 of the revised manuscript).

Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury

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Abbreviation:

LPO, lipid peroxidation; PARP, poly (ADP-ribose) polymerase; NAC, *N*-acetylcysteine; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; GSH, glutathione.

Abstract:

Methylmercury (MeHg) is well-known for causing irreversible damage in the central nervous system as well as a risk factor for inducing neuronal degeneration. However, the molecular mechanisms of MeHg-induced neurotoxicity remain unclear. Here, we investigated the effects and possible mechanisms of MeHg in the mouse cerebrum (*in vivo*) and in cultured Neuro-2a cells (*in vitro*). *In vivo* study showed that the levels of LPO in the plasma and cerebral cortex significantly increased after administration with MeHg (50 μg/kg/day) for 7 consecutive weeks. MeHg could also decrease glutathione level and increase the expressions of caspase-3, -7, and -9, which were accompanied with Bcl-2 down-regulation and up-regulation of Bax, Bak, and P53. Moreover, treatment of Neuro-2a cells with MeHg significantly reduced cell viability, increased oxidative stress damages, and induced several features of mitochondria-dependent apoptotic signals, including increased sub-G1 hypodiploids, mitochondrial dysfunctions, and the activations of PARP, and caspase cascades. These MeHg-induced apoptotic-related signals could be remarkably reversed by antioxidant NAC. MeHg also increased the phosphorylation of ERK1/2 and p38, but not JNK. Pharmacological inhibitors NAC, PD98059, and SB203580 attenuated MeHg-induced cytotoxicity, ERK1/2 and p38 activation, MMP loss, and caspase-3 activation in Neuro-2a cells. Taken together, these results suggest that the signals of ROS-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic pathways that are involved in MeHg-induced neurotoxicity.

Keywords: Methylmercury; Neurotoxicity; Apoptosis; Oxidative stress; ERK1/2; p38

1. Introduction

Methylmercury (MeHg) is a highly lipophilic and toxic environmental contaminant. It has become an important public health problem in humans because of the growing evidence of its contamination of the human food chain, such as in fish, shellfish, and other aquatic mammals (Clarkson et al., 2003; U.S. Environmental Protection Agency (EPA) 1997). MeHg, which easily crosses the blood-brain barrier (BBB), is a potent neurotoxin interfering with the brain and nervous system (the primary target tissue for the toxic effects of MeHg), and causes irreversible neuropathophysiological disorders in mammals (Li et al., 2010; Rice and Barone, 2000). It has been clinically reported that after the MeHg disasters in Japan and Iraq, adults as well as infants and children developed severe brain dysfunction and damages (Amin-Zaki et al., 1981; Marsh, 1987). Although a previous study by our group indicated that exposure of mice to MeHg, which may be similar to the dose by ingestion in MeHg-contaminated areas, could cause abnormal neural function (Huang et al., 2008), the signaling mechanisms of MeHg-induced neurotoxic effects are not yet understood.

Oxidative stress has been implicated in a wide variety of biological reactions such as cell death or central nervous system damage (Cuello et al., 2010; Tamm et al., 2006). Exposure to MeHg (1-10 μM) has reported to induce toxic effects by production of oxidative stress, which alters cellular function and eventually results in pathophysiological injury and cell death in various cells, including neuronal cells (Garg and Chang, 2006; Yin et al., 2007). Some studies have also indicated that MeHg disrupts cellular redox homeostasis and/or antioxidant enzymes and the mitochondrial electron transport chain, via excessive generation of ROS (Sarafian, 1999; Yee and Choi, 1996), thus reducing the mitochondrial inner membrane potential by altering calcium homeostasis (Levesque and Atchison, 1991; Sirois and Atchison, 2000).

Moreover, MeHg-induced oxidative stress can inhibit glutamate uptake of astrocytes, while stimulating glutamate over efflux, which results in an excessive level of synaptic glutamate and disrupts glutamine/glutamate cycling (Aschner et al., 2007; Yin et al., 2007). These undesirable MeHg-induced biological processes ultimately lead to neuronal dysfunction and cell death and suggest an association with progressive neurodegenerative diseases (Larkfors et al., 1991; Mutter et al., 2007).

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that mediate a critical role of signal transduction in mammals. MAPKs, which can be subdivided into ERK1/2, JNK, and p38 protein, are activated by modulation of important cellular functions, including proliferation, differentiation, or respond to environmental stimuli such as apoptosis (Chang and Karin, 2001; Cowan and Storey, 2003). It has been proposed that deviation from the precise regulation of MAPK signaling pathways could cause the development of human neurodegenerative diseases (Kim and Choi, 2010). ROS-induced oxidative stress have been demonstrated to activate members of the MAPKs via phosphorylation (EI-Najjar et al., 2010; Navarro et al., 2006), and it is also implicated in cellular injuries or apoptosis during neurodegenerative disorders (Loh et al., 2006). Recently, oxidative stress-induced ERK/p38 activation has been identified in mammalian cell deaths caused by exposure to toxic chemicals (Martin and Pognonec, 2010; Navarro et al., 2006). However, the mechanism underlying the effect of MeHg-induced oxidative stress in neuronal cells contributing to apoptosis is no clear.

In this study, we attempted to explore the role of oxidative stress-mediated signaling in neuronal cell death by investigating whether ROS generation, mitochondrial dysfunction, caspase cascades, and/or ERK/p38-MAPK activation are involved in MeHg-induced apoptosis in Neuro-2a cells. Moreover, the use of the potent antioxidant *N*-acetylcysteine (NAC) at different stages confirmed the

involvement of major molecules in signaling pathways. We also explored whether exposure to MeHg (50 μg/kg), which mimics the possible exposure dose in human in MeHg-contaminated areas, and has been reported to cause the neurophysiological dysfunction (Huang et al., 2008), would generate LPO, deplete GSH levels, and alter apoptotic-related gene expressions in the cerebral cortex of mice.

2. Materials and Methods

2.1. Cell Culture

Murine neuroblastoma cell line: Neuro-2a (CCL-131, American Type Culture Collection, Manassas, VA, USA) was culture in a humidified chamber with a 5% CO2–95% air mixture at 37 °C and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco/Invitrogen, Carlsbad, CA, USA), and all assays were conducted within 10-15 cell passages after unfreezing the cells from the liquid nitrogen (to keep the best morphological and biological characteristics).

2.2. Cell viability

Neuro-2a cells were seeded at 2×10^4 cells/well in 96-well plates and allowed to adhere and recover overnight. The cells were changed to fresh media and then incubated with MeHg (1-5 μM; Sigma-Aldrich, St. Louis, MO, USA) in the absence or presence of NAC (1 mM) or specific MAPK inhibitors (20 μM, Sigma-Aldrich) for 24 h. After incubation, the medium was aspirated and fresh medium containing 30 μL of 2 mg/mL 3-(4, 5-dimethyl thiazol-2-yl-)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) was added. After 4 h, the medium was removed and replaced with blue formazan crystal dissolved in dimethyl sulfoxide (100 μL; Sigma-Aldrich). Absorbance at 570 nm was measured using a microplate reader (Bio-Rad, model 550, Hercules, CA, USA).

2.3. Determination of reactive oxygen species (ROS) production

ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe: 2', 7'-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Inc, Eugene, OR, USA), as described Chen et al., 2010. In brief, cells were

coincubated with 20μM DCFH-DA at 37 ℃. After incubation with the dye, cells were resuspended in ice-cold phosphate buffered saline (PBS) and placed on ice in a dark environment. The intracellular peroxide levels were measured by flow cytometer (FACScalibur, Becton Dickinson, Sunnyvale, CA), that emitted a fluorescent signal at 525 nm. Each group was acquired more than 10000 individual cells.

2.4. Analysis of intracellular GSH contents

Neuro-2a cells were seeded at 2×10^5 cells/well in a 24-well plate and allowed to adhere and recover overnight. The cells were changed to fresh media and incubated with MeHg in the absence or presence of NAC (1 mM, Sigma-Aldrich) for 24 h. Then, cells were washed twice with PBS, and then a new medium which contained 60 μM monochlorobimane (mBCL, a sensitive fluorescent probe, Sigma-Aldrich) was added and incubated for further 30 min at 37 ℃. After loading the culture cells with mBCL, the supernantants were discarded, cells were washed twice with PBS, and the measurement the intracellular GSH levels were performed as described previously (Yen et al., 2007).

2.5. Flow cytometric analysis of sub-G1 DNA content

Cells were seeded (in the same manner as for intracellular GSH analysis) and incubated with MeHg. After 24 h incubation, the cells were detached, collected, and washed with PBS, and the analysis of sub-G1 DNA content was performed as described previously (Lu et al., 2011). The cells were subjected to flow cytometry analysis of DNA content (FACScalibur, Becton Dickinson). Nuclei displaying hypodiploid, sub-G1 DNA contents were identified as apoptotic. The sample of each group was collected more than 10000 individual cells.

2.6. Determination of Mitochondrial Membrane Potential(MMP)

Cells were seeded (in the same manner as for intracellular GSH analysis) and exposure to MeHg in the absence or presence of NAC (1 mM) or specific MAPK inhibitors (20 μM, Sigma-Aldrich). After 6 or 24 h incubation, cells were loaded with 100 nM 3,3`-di-hexyloxacarbocyanine iodide ($DiOC_6$, Molecular Probes, Inc) or 30 min at 37 ℃, and then trypsinized, collected, and washed twice with PBS. MMP was analyzed by FACScan flow cytometer (excitation at 475 nm and emission at 525 nm, Becton Dickinson)(Chen et al., 2006).

2.7. Measurement of Caspase-3 Activity

Neuro-2a cells were seeded (in the same manner as for intracellular GSH analysis) and exposure to MeHg in the absence or presence of NAC (1 mM, Sigma-Aldrich) or specific MAPK inhibitors (20 μM, Sigma-Aldrich). After 24 h incubation, the cells were lysed and determined caspase-3 activity using the CaspACE^{TM} fluorometric activity assay (Promega Corporation, Madison, WI, USA) as previously described (Lu et al., 2011). Protein levels of cell lysate samples were determined using the bicinchoninic acid protein assay kit with an absorption band of 570 nm (Pierce, Rockford, IL, USA) to normalize the cell numbers between control and different MeHg-treated groups.

2.8. Western blot analysis

Neuro-2a cells were seeded at 1×10^6 cells/well in a 6-well plate and allowed to adhere and recover overnight. The cells were changed to fresh media and incubated with MeHg in the absence or presence of NAC (1 mM, Sigma-Aldrich) or specific MAPK inhibitors (20 μM, Sigma-Aldrich) for different time intervals. After treatment, the expression of protein activation or phosphorylation was evaluated by the standard

protocols performance of Western blot (as previously described in Chen et al., 2010) using the specific antibodies, including: anti-cleaved caspase-3, -7, and -9, PARP, phosphor-p38 and phosphor-ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA, UAS), anti-Bcl-2, Bax, p38, and ERK1/2 (Santa Cruz Biotechnology, Inc., CA, USA), and α-tubulin (Epitomics, Inc., Burlingame, CA, USA).

2.9. Animal preparation

Randomly bred, normal male ICR mice (4weeks old, 20-25 g) were obtained from the Animal Center of College of Medical, National Taiwan University. All experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC), and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of China Medical University (http://cmurdc.cmu.edu.tw/LA/index.php). Mice were randomly assigned to pretreatment groups, weighed, and administrated MeHg or vehicle (oral application by gavaged). These groups were given 0 and 50 μg/kg/day MeHg in the absence or present of NAC (150 mg/kg/day) for 7 consecutive weeks. Each group contained more than 12 mice (n = 12~15). All experimental mice were deep anesthesia by an intraperitoneal injection of pentobarbital (80 mg/kg) and the whole blood samples were collected from an eyehole vessel. Whole blood sample were centrifuged at 3,000 × g for 10 min, and plasma was obtained, and LPO levels was assayed immediately. At the same time, mice were sacrificed by decapitation under pentobarbital anesthesia, and the cerebral cortex were quickly removed and stored at liquid nitrogen until use, and then was analysis of LPO levels, GSH content, and apoptosis-related genes expression.

2.10. Lipid peroxidation(LPO) analysis

Neuro-2a cells were seeded (in the same manner as for Western blot analysis) and exposure to MeHg alone or in combination with NAC (1 mM, Sigma-Aldrich). After 24 h incubation, cells were harvested and homogenized in ice-cold 20 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM butylated hydroxytoluene to prevent sample oxidation. The cerebral cortex was weighted and homogenized separately in ice-cold 20 mM Tris-HCl buffer, pH 7.4 (100 mg tissue/ml buffer), then homogenized sample was assayed immediately. LPO levels of equal volumes sample were measured using a commercial LPO assay kit (Calbiochem, La Jolla, CA, USA) as described by Huang et al. (2008) and Lu et al. (2010). The protein concentration of each sample was determined using the bicinchoninic acid protein assay kit (Pierce). LPO level was expressed as nanomoles (nmol) MDA per milligram protein and estimated from the standard curve.

2.11. Measurement of glutathione (GSH) levels in the cerebral cortex

The cerebral cortex was homogenated with an isotonic buffer (25 mM Hepes. pH 7.4, containing 250 mM sucrose) and then centrifuged at 1,000 × *g* at 4 ℃. Each sample was discarded the pellet and added 10× lysis buffer to the supernant, and placed on ice for 10 min. After centrifuged, the supernant was performed the measurement of GSH levels using Glutathione assay kit (Sigma-Aldrich) according to the manufacturer's instructions. The protein concentration of each sample was determined using the bicinchoninic acid protein assay kit (Pierce). GSH level was expressed as nanomoles (nmol) GSH per microgram protein and estimated from the standard curve.

2.12. Real-time quantitative reverse-transcribed polymerase chain reaction (RT-PCR)

analysis

The expression of apoptosis-related genes was evaluated by real-time quantitative RT-PCR, as previously described (Lu et al., 2010). Briefly, intracellular total RNA was extracted from the cerebral cortex using RNeasy kits (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using the AMV RTase (reverse transcriptase enzyme; Promega Corporation, Pty. Ltd.) according to the manufacture's instructions. Each sample (2 μ1 cDNA) was tested with Real-time Sybr Green PCR reagent (Invitrogen, USA) with mouse specific primers (PCR primers for the examined genes were listed in Table 1.) in a 25-μl reaction volume, and amplification was performed using an ABI StepOnePlus sequence detection system (PE, Applied Biosystems, CA, USA). Data analysis was performed using StepOne^{TM} software (Version 2.1, Applied Biosystems). All amplification curves were analyzed with a normalized reporter (R_n: the ratio of the fluorescence emission intensity to the fluorescence signal of the passive reference dye) threshold of 0.2 to obtain the C_T values (threshold cycle). The reference control genes were measured with four replicates in each PCR run, and their average C_T was used for relative quantification analyses (the relative quantification method utilizing real-time PCR efficiencies (Pfaffl et al., 2002)). TF expression data were normalized by subtracting the mean of reference gene C_T value from their C_T value (ΔC_T). The Fold Change value was calculated using the expression $2^{-\Delta\Delta C}{}_T$, where $\Delta\Delta C_T$ represents $\Delta C_{T\text{-condition of interest}} - \Delta C_{T\text{-control}}$. Prior to conducting statistical analyses, the fold change from the mean of the control group was calculated for each individual sample.

2.13. Statistical analysis

Data are presented as means ± standard deviations (S.D.) The significance of difference was evaluated by the Student's t-test. When more than one group was

compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) was used for analysis, and the Duncans's post hoc test was applied to identify group differences. The P value less than 0.05 was considered to be significant. The statistical package SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. MeHg exposure induces LPO, depletion of GSH levels, and changes in apoptosis-related gene expression in the cerebral cortex of mice

Our previous study indicated that exposure to MeHg (50 μg/kg/day) caused neurophysiological dysfunctions and biological changes in mice brain, which was accompanied with significant mercury accumulation (Huang et al., 2008). However, the toxicological effects and possibly molecular mechanism of MeHg-induced neurotoxicity have not been understood. Therefore, we investigated the LPO and GSH levels in the plasma and/or cerebral cortex of MeHg-treated mice (50 μg/kg/day, by oral gavage). The results revealed that LPO levels in the plasma and cerebral cortex significantly increased after MeHg exposure for 7 consecutive weeks (Supplement Figure 1), whereas the measurement of GSH levels in the cerebral cortex markedly decreased (Figure 1).

We further investigated whether MeHg-induced oxidative stress could cause apoptosis in the cerebral cortex of mice. To address this issue, we analyzed apoptosis-related gene expression by using real-time quantitative RT-PCR. As shown in Figure 2, there was significant down-regulation of Bcl-2 (anti-apoptotic gene) and up-regulation of Bax, Bak, and p53 (pro-apoptotic genes) expression in the cerebral cortex of mice after MeHg exposure for 7 consecutive weeks (Figure 2A). Furthermore, these changes were accompanied by marked up-regulation of caspase-3, caspase-7, and caspase-9 expressions (Figure 2B). Antioxidant NAC (150 mg/kg/day) effectively prevented MeHg-induced toxic responses (Supplement Figure 1, Figures 1 and 2).

3.2. MeHg induces cell death, ROS production, and intracellular GSH depletion in

Neuro-2a cells

To investigate whether the mechanisms involved in MeHg-induced neurotoxicity, we explored the *in vitro* effects of MeHg in Neuro-2a cells. The cell viability of Neuro-2a cells was markedly decreased after 24 h MeHg (1-5 μM) treatment, and the LD_{50} was determined to be approximately 3 μM (Figure 3A).

To further evaluate the effects of MeHg on oxidative stress damage, we treated the cells with MeHg (3 and 5 μM) and measured ROS generation, LPO production, and intracellular GSH levels. After exposure of Neuro-2a cells to MeHg for 0.5-2 h, the intracellular ROS levels were found to be significantly increased by using DCF fluorescence probe as an indicator of ROS formation (Figure 3B). Incubation of cells with MeHg for 24 h also produced remarkably high malondialdehyde (MDA) levels in the cell membrane (3 μM MeHg, 3.08 ± 0.07; 5 μM MeHg, 3.91 ± 0.08; control, 1.92 ± 0.08 nmole-MDA/mg protein)(Figure 3C). Moreover, the levels of intracellular GSH (a principal cellular protective thiol against oxidative stress-induced toxicity, and determined using an mBCl fluorescent probe) was significantly decreased after treatment with MeHg (3 and 5 μM, not 1 μM) for 24 h (3 μM MeHg, 61.31 ± 3.13%; 5 μM MeHg, 30.60 ± 4.07% of control)(Figure 3D). These MeHg-induced responses could be reversed by NAC (1 mM) (Figures 3B, 3C, 3D, and 3E).

3.3 MeHg causes cell death via mitochondria-dependent apoptosis pathways in Neuro-2a cells

To investigate the involvement of apoptosis in MeHg-induced Neuro-2a cell cytotoxicity, we analyzed the sub-G1 hypodiploid cell population (as an indicator of apoptosis) by flow cytometry. Cells treated with MeHg (3 and 5 μM) for 24 h exhibited a significant increase in the sub-G1 hypodiploid cell population (Figure 4A). Moreover, caspase-3 activity (an integral step in the majority of apoptotic events) was

also markedly induced after treatment of Neuro-2a cells with MeHg (Figure 4B). These results indicated that exposure of Neuro-2a cells to MeHg could induce apoptosis.

Next, we explored whether MeHg-induced apoptosis was mediated through the mitochondrial dysfunction. To show that MeHg affected mitochondrial permeability transition, MMP was analyzed using flow cytometry with the cationic dye $DiOC_6$. As shown in Figure 5A, exposure of Neuro-2a cells to MeHg (3 μM) markedly induced MMP loss (for 6 and 24 h). We also investigated cytochrome c release from the mitochondria into the cytosol in MeHg-treated Neuro-2a cells. Cells exposure to MeHg (3 μM) for 6 h effectively increased cytochrome c release in the cytosol fraction, and this increase in the level of cytochrome c was more significant after 24 h MeHg exposure (Figure 5B). Antioxidant NAC (1 mM) could effectively reverse the MeHg-induced responses (Figures 5A and B). Moreover, we further examined the changes in the expression of Bcl-2 family proteins. Treatment of Neuro-2a cells with MeHg (3 μM) significantly decreased the expression of Bcl-2 and increased the level of Bax, which led to a marked shift in the pro-apoptotic (Bax)/anti-apoptotic (Bcl-2) expression ratio toward an apoptosis-associated state (Figure 5C).

To further investigate whether MeHg-induced the activation of cysteine proteases, which are important biomarkers of the apoptotic process representing both the initiation and execution of cell death, the expressions of caspase cascades were detected by Western blot analysis. As shown in Figure 5D, it increased the activation of caspase-3 and caspase-7 in Neuro-2a cells treated with MeHg (3 μM) for 6-24h. The MeHg-induced caspase-3 activity in Neuro-2a cells could be reversed by NAC (Figure 4B). MeHg also significantly increased the level of cleaved product (active form) of PARP as well as the expression of upstream caspase-9 (Figure 5D).

3.4. Effects of MeHg on activation of ERK- and p38-MAPK in Neuro-2a cells

To further evaluate the involvement of MAPKs signals in responses triggered by MeHg-induced apoptosis, the expressions of MAPKs activation were examined. As shown in Figure 6A, exposure of neuro-2a cells to MeHg (3 μM) significantly increased the levels of phosphorylation of ERK1/2- and p38-MAPK (for 0.5-2 h), but not that of JNK. To determine the relationship between MeHg-induced apoptotic signaling transduction in Neuro-2a cells and MAPKs activation, the cells were pretreated with the specific ERK inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125. It was found that MeHg-induced neuronal cell cytotoxicity was attenuated by the ERK1/2 and p38 inhibitors (20 μM), but not by the JNK inhibitor (Figure 6B, *a*). NAC, ERK and p38 inhibitors could prevent MeHg-induced ERK1/2- and p38-MAPK activation (Figure 6B, *b* and *c*). Loss of MMP and the increase of caspase-3 activity induced by MeHg-treated Neuro-2a cells could also be effectively reversed by ERK and p38 inhibitors (Figures 6C and D). Furthermore, MeHg-induced increase in ERK1/2- and p38-MAPK protein phosphorylation was observed in the cerebral cortex of MeHg-treated mice (50 μg/kg/day, for 7 consecutive weeks), which could be prevented by NAC (Supplement figure 2). These results indicate that ROS-mediated ERK1/2 and p38-MAPK activation play a crucial role in MeHg-induced neuronal cell apoptosis.

4. Discussion

Many studies have indicated that MeHg is a potent neurotoxicant affecting both the developing and mature central nervous system, and can cause severe neuropathophysiological disorders with exposure to high concentrations (0.5-40 ppm in drinking water or 0.2-2 mg/kg/day, for more than 7 consecutive days) *in vivo* system, including loss of neurons in the calcarine cortex, cerebellar Purkinje, and granule cells, leading to vision, motion, or postural abnormalities (Carvalho et al., 2007; Chuu et al., 2007; Dare et al., 2003; Goulet et al., 2003; Onishchenko et al., 2007). Exposure to MeHg (20-50 μg/kg) in mice, which was the possible dosage from food ingested in MeHg-contaminated areas (8.03-174 μg-MeHg/kg, even more than 200 μg-MeHg/kg), caused severe neurotoxic injuries has also reported, that accompanied with significant mercury accumulation (113.0-241.8 ng-mercury/g wet. wt.) and oxidative stress generation in the brain regains (Grandjean et al., 1992; Huang et al., 2008 and 2011; Maramba et al., 2006; Qiu et al., 2008). Oxidative stress, which has demonstrated to strongly induce under MeHg-exposed conditions and play a key role for cascade activation during mercury-induced injury, is involved in the progression of brain and/or neuronal cell dysfunction and death in mammals (Dreiem et al., 2005; Shanker et al., 2004 Yin et al., 2007). Of late, an increasing number of studies have suggested that oxidative stress, which disturbs the physiological functions of neuronal cells and causes apoptosis, is linked to the progression of neurodegenerative diseases (Branham et al., 2004; Loh et al., 2006), and clinical studies also described that significant and higher concentrations of mercury are detected in the blood and/or brain regions of Alzheimer's disease patients compared to healthy peoples; these suggest a decisive role of mercury in the origin or progression of neurodegenerative diseases (Gerhardsson et al., 2008; Hock et al., 1998; Mutter et

al., 2007). Despite several studies showing that MeHg can produce neurotoxic injuries by inducing oxidative stress in mammals, the role of ROS and the precise signaling mechanisms underlying MeHg-induced neuronal degeneration and cell death are still unclear. In this study, our results revealed that MeHg significantly induced LPO production (as an indicator for oxidative stress damage formation) in the plasma and cerebral cortex of mice exposed to MeHg (50 μg/kg/day) for 7 consecutive weeks, which was accompanied with GSH depletion. Meanwhile, the results also revealed that MeHg significantly altered apoptosis-related genes expression, including anti-apoptotic (Bcl-2), pro-apoptotic (Bax, Bak, and p53), and caspase cascades (caspase-3, caspase-7, and caspase-9) in the cerebral cortex of MeHg-treated mice. Moreover, MeHg (1-5 μM) exposure significantly decreased cell viability, increased ROS production, depleted intracellular GSH, and caused apoptotic events (increase in sub-G1 hypodiploid cell population and caspase cascades activation) in Neuro-2a cells. The antioxidant NAC could effectively prevent, but not fully reverse, these MeHg-induced responses. These findings indicate that oxidative stress is caused by MeHg exposure, and it may be involved in MeHg-induced neuronal degeneration and cell apoptosis. Furthermore, Fujimura et al (2009) has reported that the expression of Rac1 (Rho-family protein) can be down-regulated and ultimately led to apoptosis in MeHg-exposed neuronal cells. Tofighi et al (2011) has also indicated that MeHg induces caspase-independent cell death via parallel activation of calpains and lysosomal proteases in hippocampal neurons. However, the critical roles of these signals in MeHg-induced neurotoxicity will still investigation in the future.

ROS can elicit oxidative stress, which triggers cell death; thus, it can be implicated in various neurodegenerative conditions resulting from metal-induced neurotoxicity. (Bush, 2000; Rana, 2008). ROS is also known to cause mitochondrial dysfunction (as being a key mechanism in apoptosis) by oxidative stress-induced

apoptosis (Chen et al., 2006, Lu et al., 2011). Mitochondria are highly sensitive to the effects of oxidative stress, and 2 major events have been noted in oxidative stress-induced apoptosis involving mitochondrial dysfunction. One of the events is alteration in the mitochondrial membrane permeability and the subsequent depolarization of MMP; while the other event is the release of mitochondria-associated proteins (including cytochrome c, Apaf-1, and apoptosis-inducing factor) from the intermembrane space of mitochondria into the cytosol (Chen et al., 2010; Kroemer et al., 1997; Lu et al., 2010). Moreover, the biological function of Bcl-2 family proteins has been demonstrated to regulate mitochondrial-dependent apoptosis while balancing anti- and pro-apoptotic members to arbitrate life/death decisions. Therefore, the ratio of Bcl-2 to Bax is a pivotal factor that determines whether apoptosis will occur in the cells exposed to injurious chemicals (Cheng et al., 2007; Pradelli et al., 2010). Here, we found that MeHg could capable of inducing apoptotic cell death in Neuro-2a cells, which was accompanied with trigger MMP depolarization and cytochrome c release. Moreover, treatment with 3 μM MeHg significantly increased Bax and decreased Bcl-2 protein expression in Neuro-2a cells, and resulted in an increase of Bax/Bcl-2 ratio that might contribute to the promotion of MeHg-induced apoptosis. These MeHg-induced neuronal cells apoptotic responses could be prevented by the antioxidant NAC. Therefore, these results implicate that MeHg-induced oxidative stress-regulated Neuro-2a cell apoptosis involves in the mitochondria-dependent apoptotic pathway.

MAPKs, including ERK1/2, JNK, and p38, play critical roles as mediators of cellular responses to extracellular stimuli, such as the proliferation, differentiation, survival, and death of nervous cells (Chang and Karin, 2001; Chen et al., 2009; Cowan and Storey, 2003). MAPKs activation is also involved in apoptosis and may play a pivotal role in the progress of neurodegenerative diseases (Kim and Choi, 2010;

Miloso et al., 2008). Some studies have indicated that oxidative stress is an important risk factor in the development of Alzheimer's disease via MAPK signaling activations and downstream-regulated apoptosis in neuronal cells exposed to many injurious agents (Margues et la., 2003; Puig et al., 2004). To our knowledge, however, a limited number of studies have investigated the role of MAPKs in MeHg-induced neuronal cell apoptosis. In the present study, we found that MeHg induced the activation of ERK1/2- and p38-MAPK, but not that of JNK, in Neuro-2a cells. Pretreatment of cells with the antioxidant NAC, specific ERK inhibitor PD98059, and p38 inhibitor SB203580, but not JNK inhibitor SP600125, attenuated MeHg-induced cytotoxicity and the phosphorylation levels of ERK1/2- and p38-MAPK protein expression. It also could significantly reversed MeHg-induced depolarization of MMP and the increase in caspase-3 activation by pretreated with NAC and the specific ERK and p38 inhibitors. Moreover, the effects of MeHg-induced ERK1/2- and p38-MAPK activation were revealed in the cerebral cortex of mice exposed to MeHg (50 μg/kg/day, for 7 consecutive weeks), which could be prevented by NAC. Thus, these findings demonstrate that ROS-triggered ERK1/2- and p38-MAPK activated pathway mediate MeHg-induced neuronal cell apoptosis.

5. Conclusion

In conclusion, the present *in vivo* and *in vitro* results provides evidence that MeHg is capable of inducing neuronal degeneration and apoptosis, and more importantly, reveals the molecular basis for its effects. This study has clearly demonstrated that MeHg-induced oxidative stress causes apoptosis in neuronal cells through ERK1/2- and p38-MAPK activation regulated mitochondrial dysfunction triggered PARP and caspase-9 activation, and involved in caspase-3/-7-mediated mechanism (Figure 7). These observations further clarify the neurotoxic mechanisms of MeHg, and provide beneficial evidence to suggest that MeHg-induced neurotoxicity may be an important risk factor for neurodegenerative diseases.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

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Figure Legends:

Figure 1. Influence of MeHg on GSH levels of cerebral cortex in MeHg-exposed mice. The cerebral cortex of mice treated with MeHg (50 μg/kg/day) for 7 consecutive weeks in the absence or presence of NAC (150 mg/kg/day) was detected GSH levels as described in Materials and Methods. All data are presented as mean ± S.D. (n = 12~15 for each group). $^{*}p < 0.05$ as compared with vehicle control group. $^{\#}p < 0.05$ as compared with MeHg group alone.

Figure 2. MeHg treatment regulated gene expression for apoptotic factors in the cerebral cortex of mice. Mice were treated with MeHg (50 μg/kg/day) for 7 consecutive weeks in the absence or presence of NAC (150 mg/kg/day), and the expression of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax, Bak, P53)(A), and caspase-related genes (caspase-3, -7, -9)(B) of the cerebral cortex were analyzed by real-time quantitative RT-PCR using SYBR Green. Target gene expression was normalized toβ-actin, and the results are expressed as fold change from vehicle control. Results are expressed as mean ± S.D. (n = 12~15 for each group). $^{*}p < 0.05$ as compared with vehicle control group. $^{\#}p < 0.05$ as compared with MeHg group alone.

Figure 3. Effects of MeHg on cell viability and oxidative stress damage generation in Neuro-2a cells. (A) Cells were incubated with MeHg (1-5 μM) for 24 h, and cell viability was determined by MTT assay. (B) Neuro-2a cells were incubated with MeHg (3 and 5 μM) for various time courses in the absence or presence of NAC (1 mM) for 2h, and ROS was determined by flow cytometric analysis. (C) Cell

membrane LPO production, (D) intracellular GSH levels, and (E) Cell viability after incubated of Neuro-2a cells with MeHg (3 and 5 μM) for 24 h in the absence or presence of NAC (1 mM) were determined as described in the Materials and Methods section. All data are expressed as mean ± S.D. for four independent experiments with triplicate determination. *$p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with MeHg alone.

Figure 4. Analysis of sub-G1 hypodiploid cell population and caspase-3 activity showing MeHg-induced apoptosis in Neuro-2a cells. Cells were incubated with MeHg (3 and 5 μM) for 24 h in the absence or presence of NAC (1 mM). (A) Cells with genomic DNA fragmentation (Sub-G1 DNA content) were analyzed by flow cytometry, and (B) caspase-3 activity was measured by the CaspACE^{TM} fluorometric activity assay kit as described in the Materials and Methods section. Data are presented as mean ± S.D. for four independent experiments with triplicate determination. *$p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with MeHg group alone.

Figure 5. MeHg induced mitochondrial dysfunction, cleavages of PARP, and caspase cascades activation in Neuro-2a cells. Cells were incubated with MeHg (3 or 5 μM) for 6-24 h in the absence or presence of NAC (1 mM). MMP) depolarization was determined by flow cytometry (A); cytochrome c release (B), Bcl-2 and Bax protein expressions (C), and PARP cleavage and the caspase-3, -7, and -9 expressions (D) were examined by Western blot analysis as described in the Materials and Methods section. Data in A are presented as mean ± S.D. for four independent experiments with triplicate determination. Results shown in B, C and D are typical representatives of at last three independent experiments. The intensity of bands were analyzed by

densitometry and expressed as fold changes relative to the control (mean ± S.D.). *p < 0.05 as compared with vehicle control. $^{\#}p$ < 0.05 as compared with MeHg alone.

Figure 6. MeHg-triggered ERK1/2- and p38-MAPK activation in Neuro-2a cells. (A) Cells were incubated with MeHg (3 μM) for 0.5-2 h, and analyzed ERK1/2- and p38-MAPK phosphorylation by Western blotting. In addition, Neuro-2a cells were treated with MeHg (3 μM) in the absence or presence of EKR1/2 inhibitor (PD98059, 20 μM), p38 inhibitor (SB203580, 20 μM), or JNK inhibitor (SP600125, 20 μM). Cell viability was detected by MTT assay (B-*a*, for 24 h), the phosphorylation of ERK1/2- and p38-MAPK proteins were detected by Western blot analysis (B-*b*, and -*c*, for 1 h), MMP depolarization was determined by flow cytometry (C, for 24 h), and caspase-3 activity was measured by the CaspACE^{TM} fluorometric activity assay kit (D, for 24 h) as described in the Materials and Methods section. Results shown in A, B-*b*, and B-*c* are typical representatives of at last three independent experiments, the intensity of bands were analyzed by densitometry and expressed as fold changes relative to the control (mean ± S.D.). Data in B-*a*, C, and D are presented as mean ± S.D. for four independent experiments with triplicate determination. *p < 0.05 as compared with vehicle control. $^{\#}p$ < 0.05 as compared with MeHg alone.

Figure 7. Schematic diagram of the signal pathways involving in MeHg-induced neuron cell apoptosis is illustrated. Proposed models show that MeHg causes neuron cell apoptosis through ROS-induced ERK1/2- and p38-MAPK activation regulated mitochondria-dependent signaling cascades.

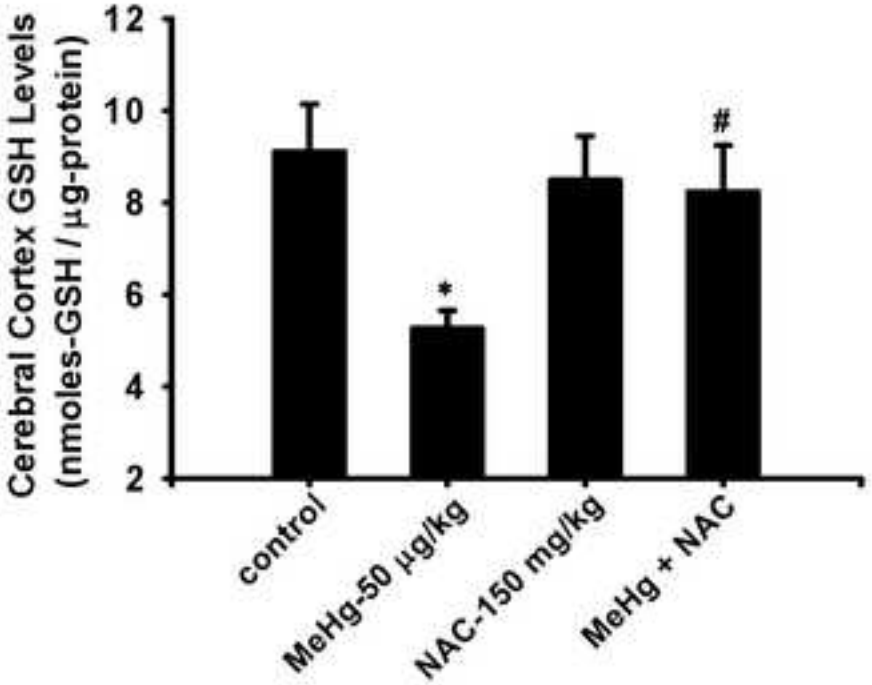
Table(s)

Table 1. Primer sequences used for the real-time quantitative RT-PCR analysis

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)	Reference
Bcl-2	TGGTCTCTATTGCAGCTCAGAC	TGGCCCAATCTAGGAAATGTTC	Harvilchuck et al, 2009
Bax	GGAATTCCAAGAAGCTGAGCGAGTGT	GGAATTCTTCTTCCAGATGGTGAGCGAG	Hoijman et al. 2004
Bak	CCCAGGACACAGAGGAGGTC	GCCCAACAGAACCACACCAAAA	Kim et al. 2004
p53	GGGACAGCCAAGTCTGTTATG	GGAGTCTTCCAGTGT GATAT	Kalia and Bansal, 2009
Caspase-3	GGAGCTGGACTGTGGCATTGA	CAGTTCTTTCGTGAGCATGGA	Wang and Han, 2009
Caspase-7	CCGAGTGCCCACTTATCTGT	ACCTGTCGCTTTGTCGAAGT	Yang et al., 2008
Caspase-9	TGCACTTCCTCTCAAGGCAGGACC	TCCAAGGTCTCCATGTACCAGGAGC	Kalia and Bansal, 2009
β-actin	TGTGATGGTGGGAATGGGTCAG	TTTGATGTCACGCACGATTTCC	Min et al. 2009

Figure(s)

Figure 1.



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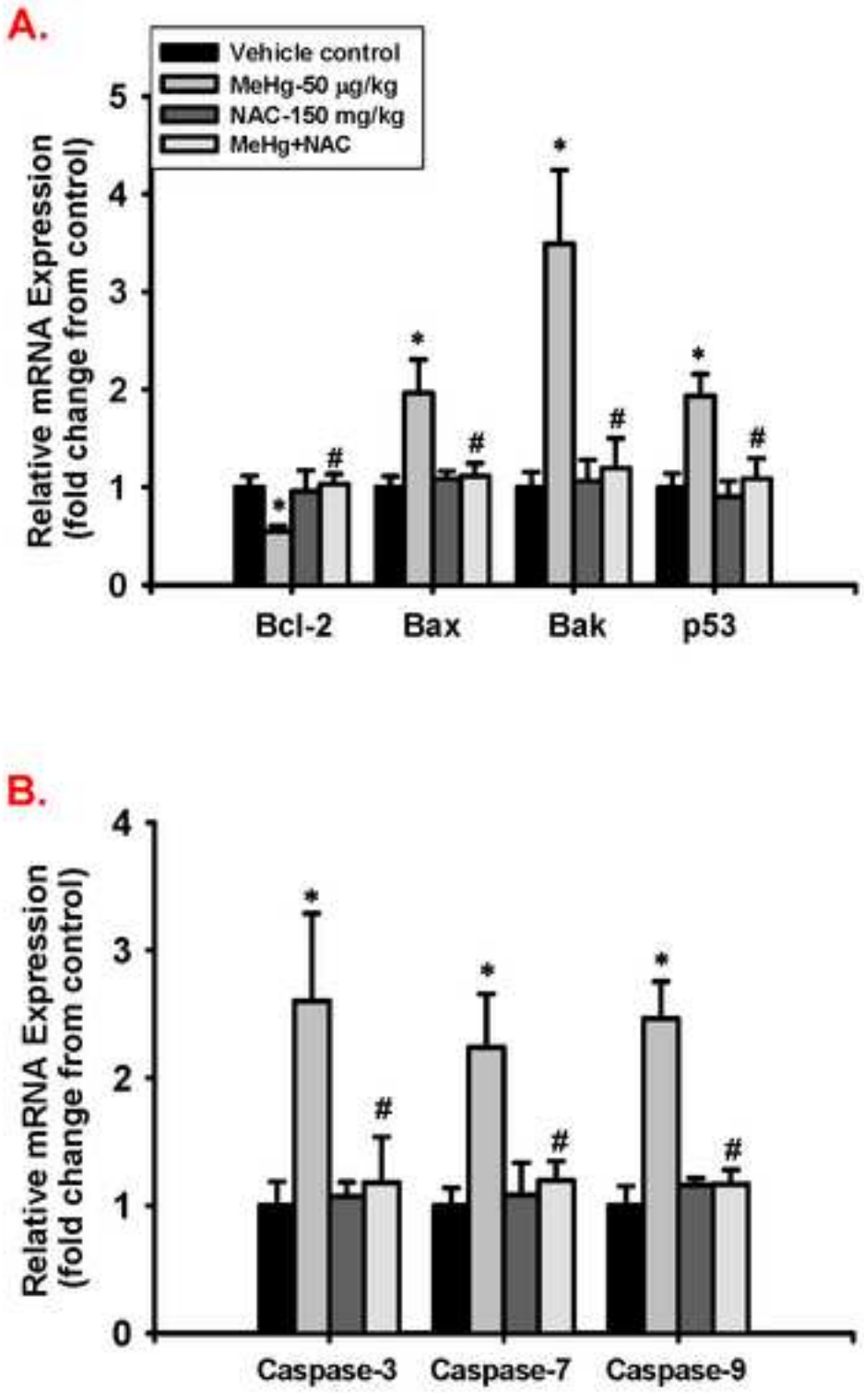

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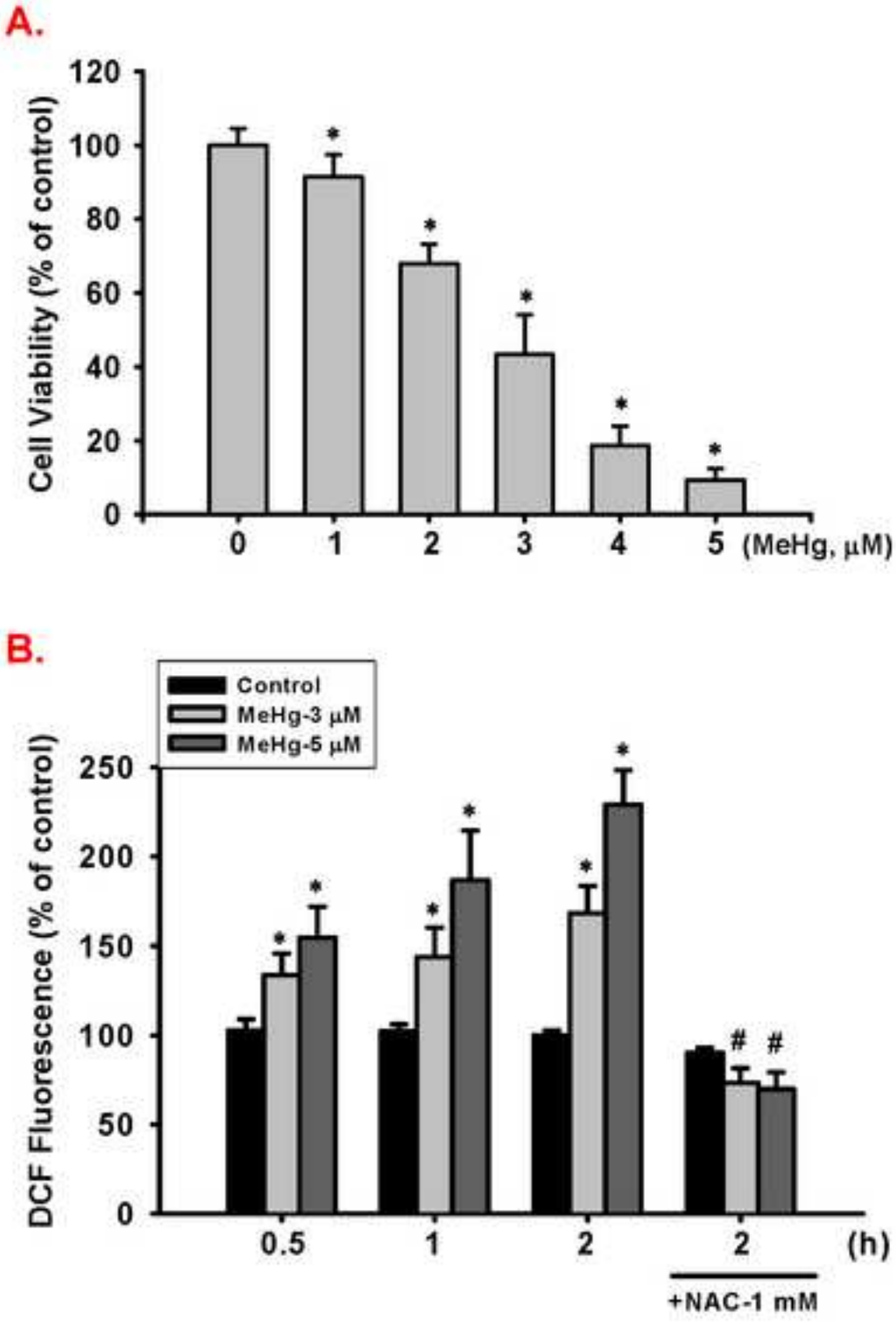

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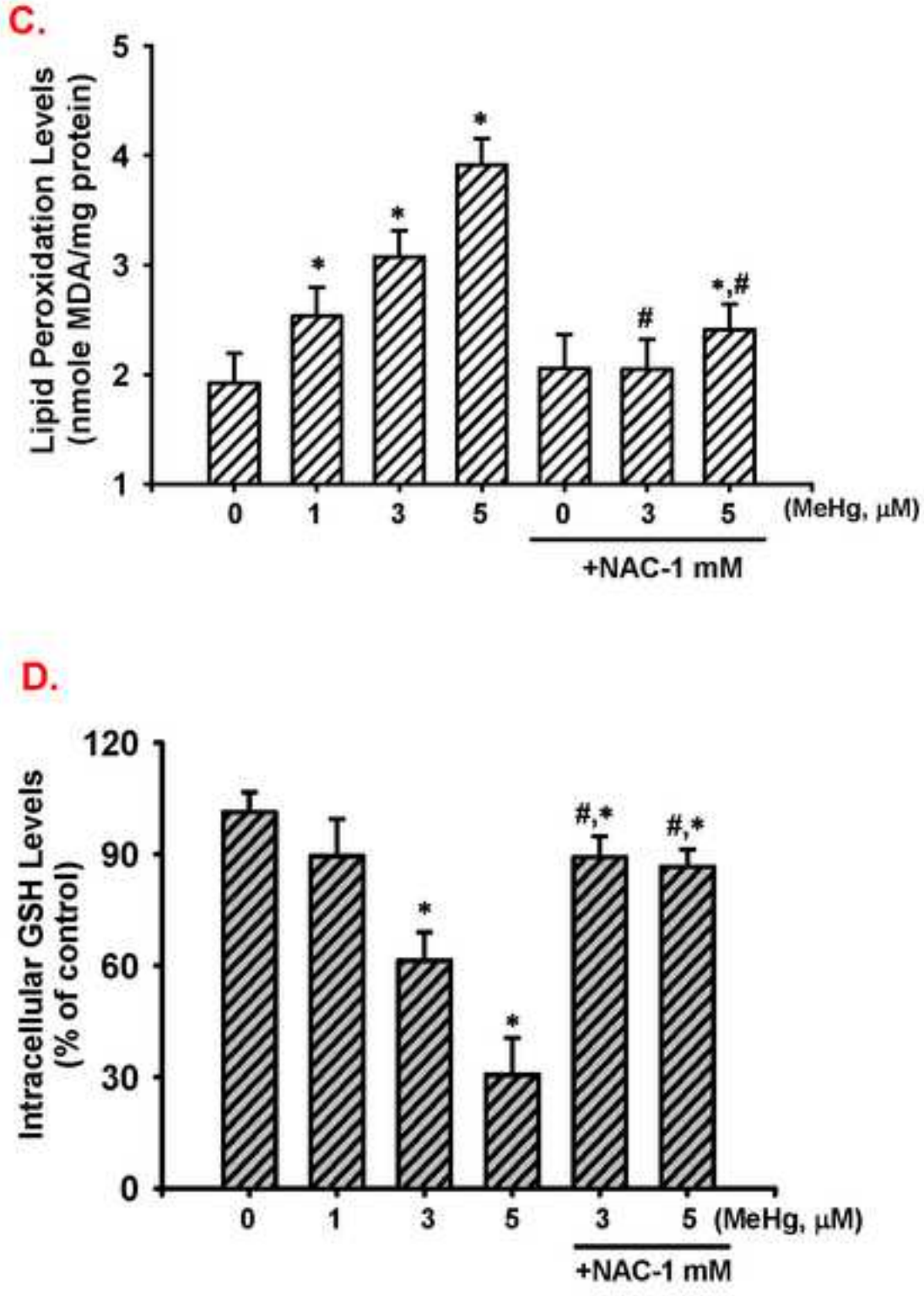

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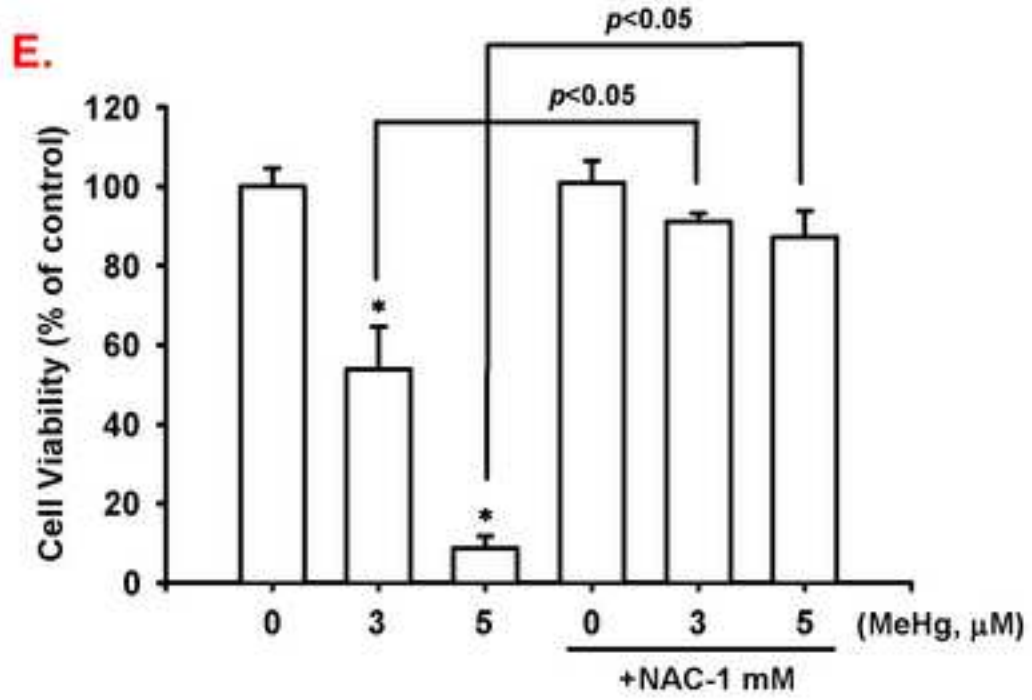

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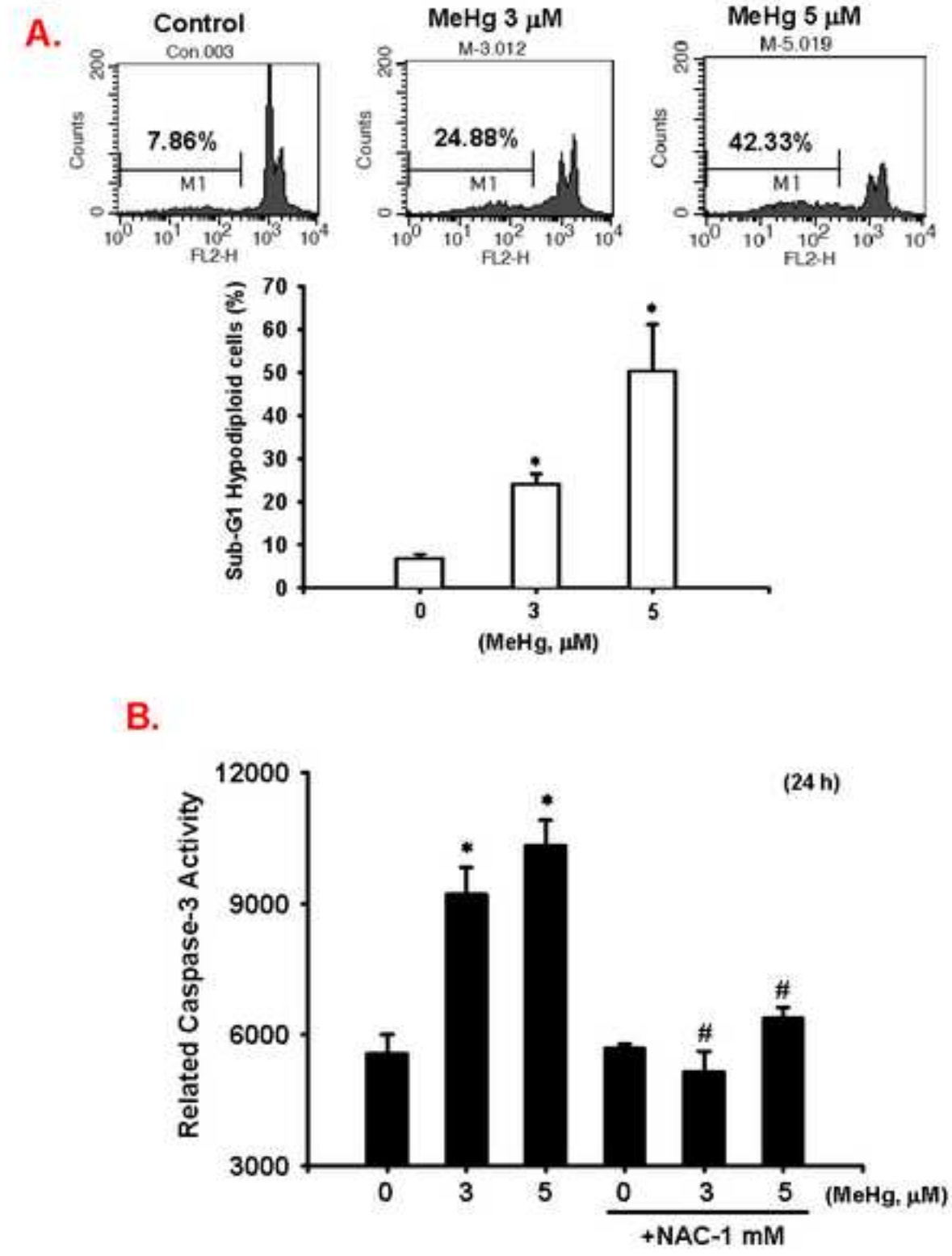

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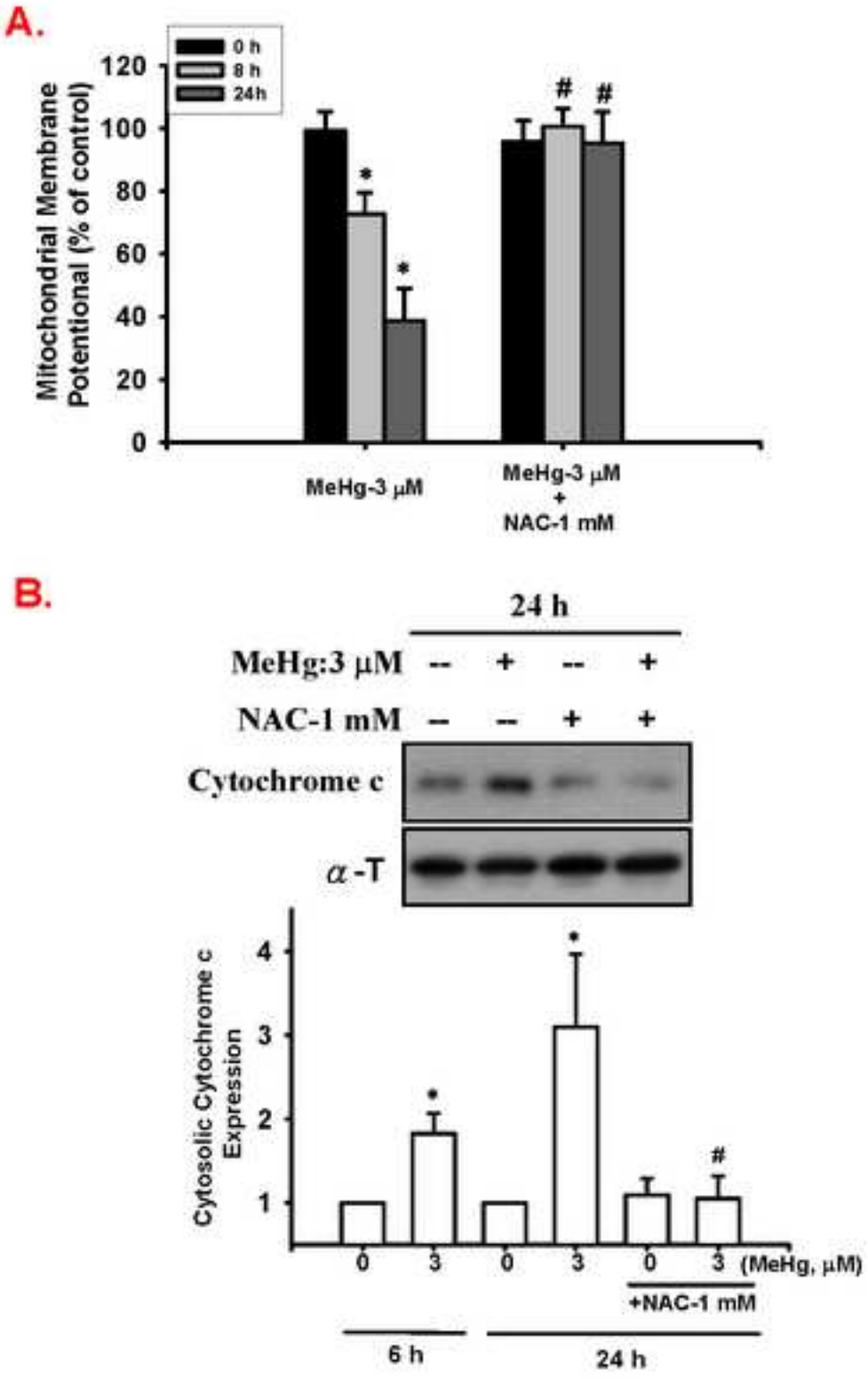

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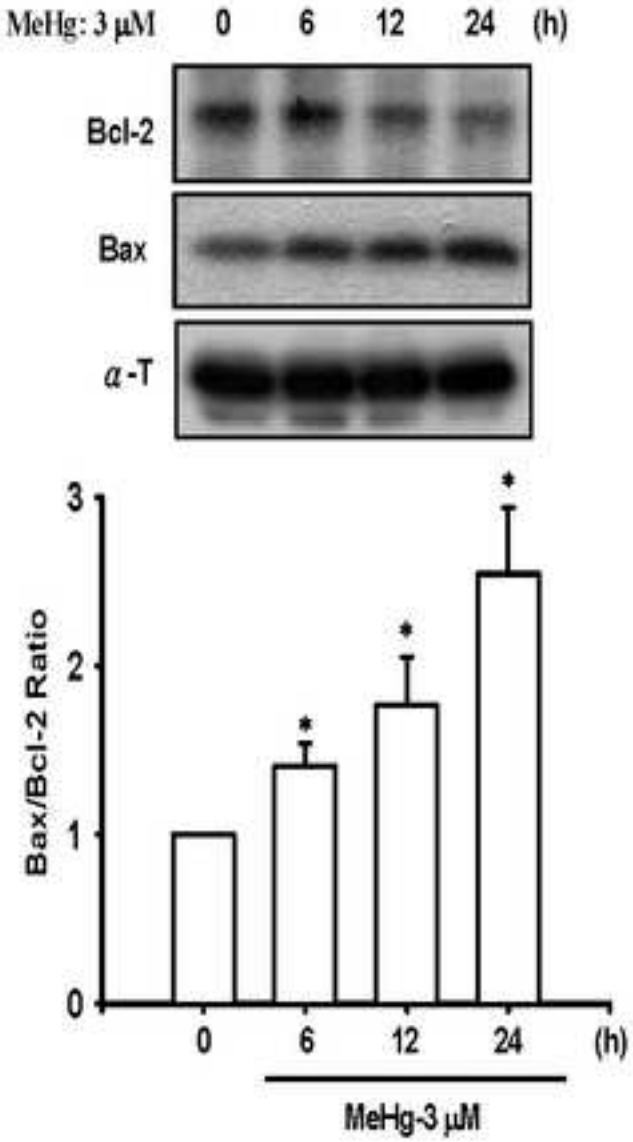

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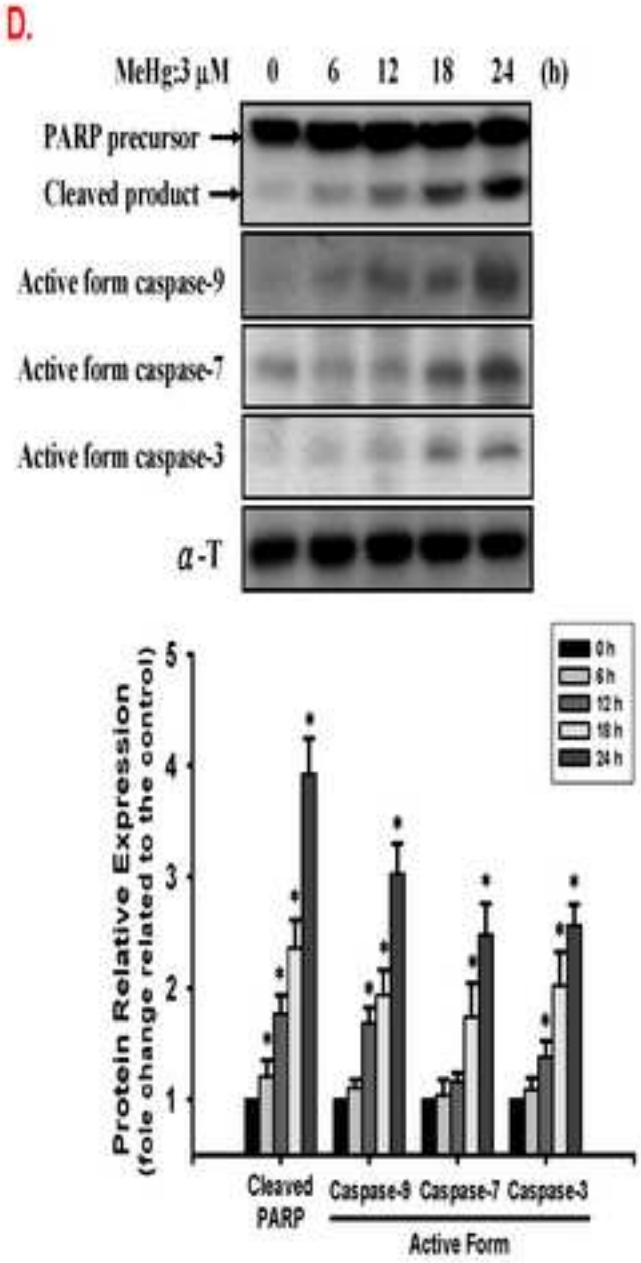

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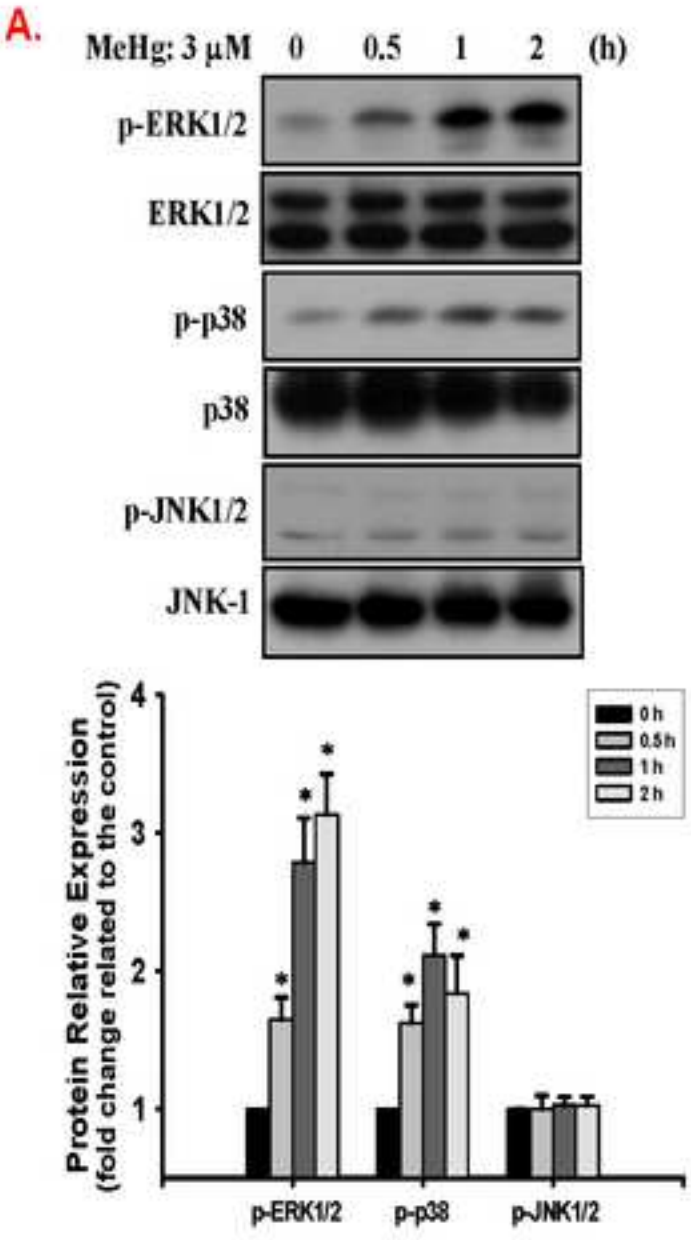

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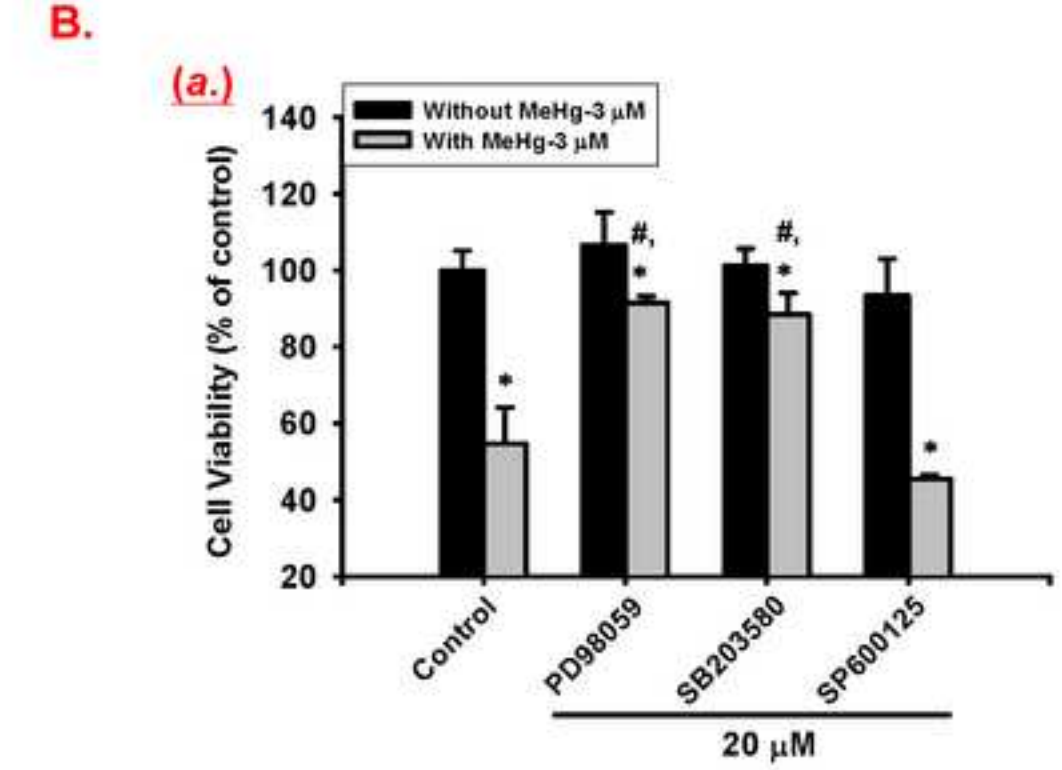

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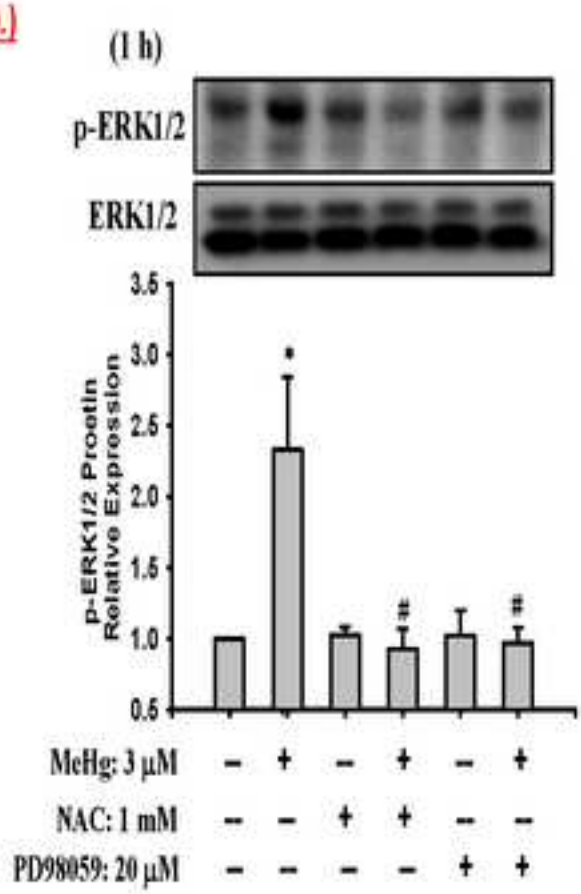
Figure 6.

B. (b.)



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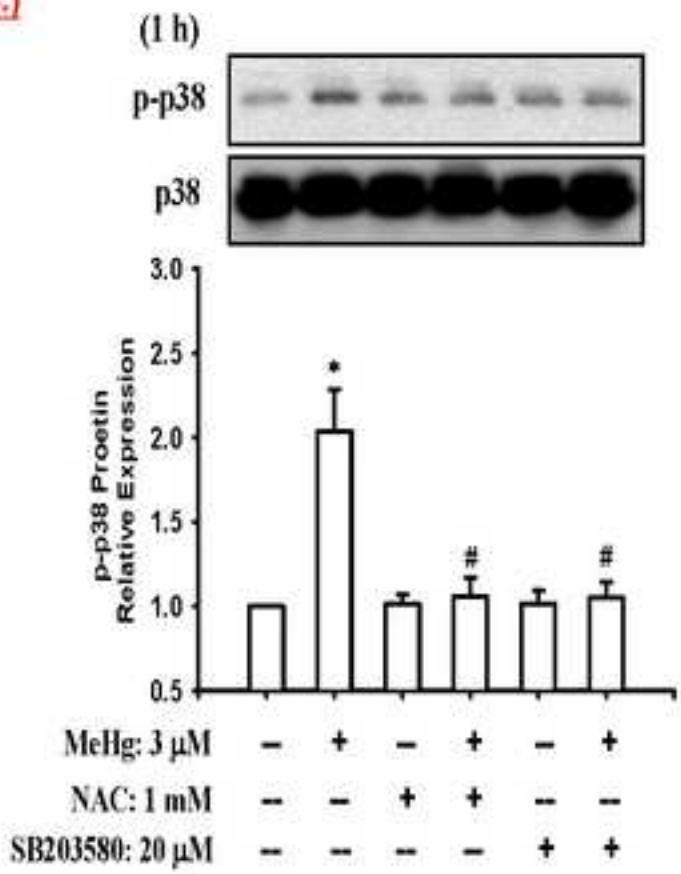

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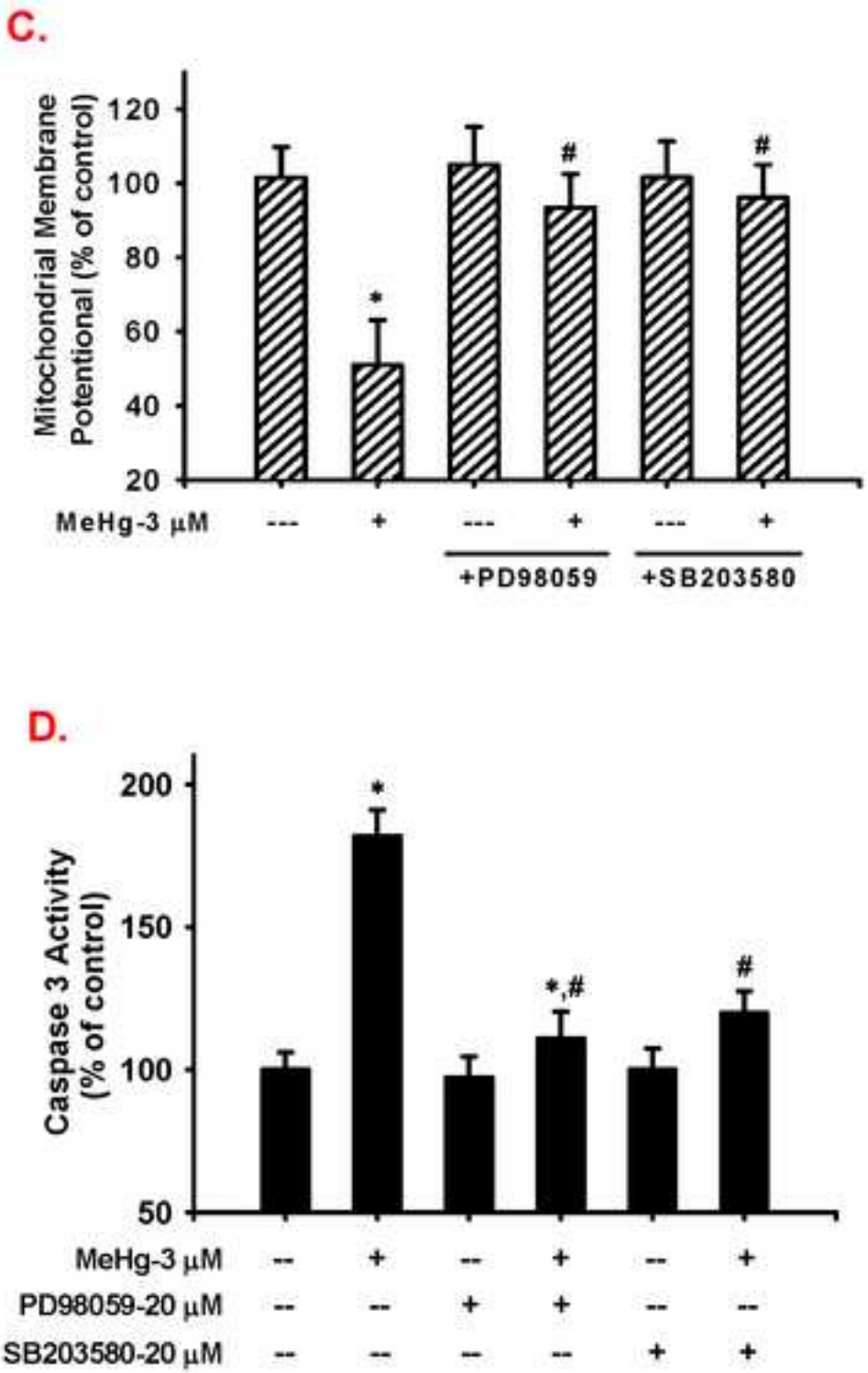

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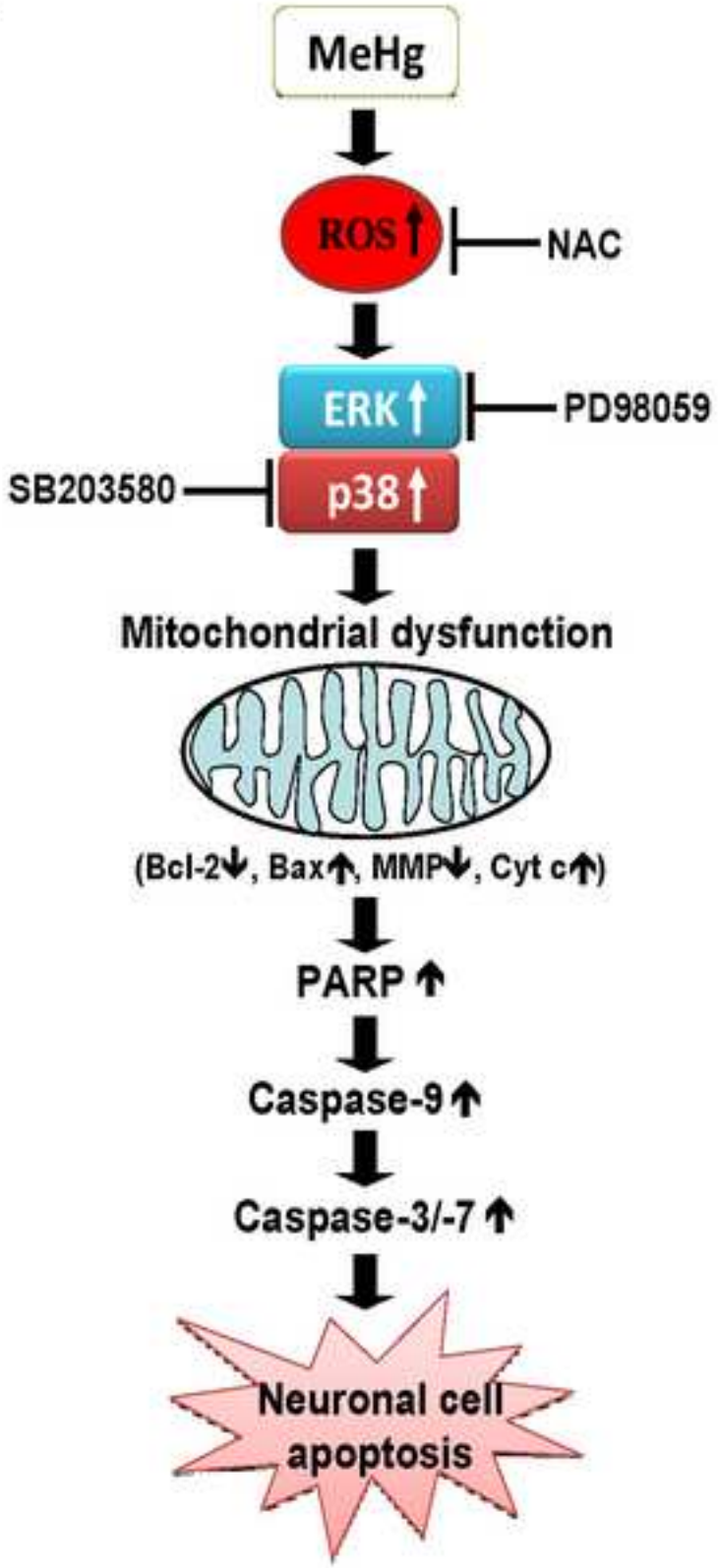

Supplementary

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