学 位 論 文 の 要 旨

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学 位 論 文 題 名

Ameliorative mechanism of dietary components on cadmium or mercury-induced toxicities in PC12 cells

（PC12 細胞におけるカドミウムまたは水銀によって引き起こされる毒性に対する 食物成分の改善メカニズム）

Environmental pollution is one of the greatest challenges faced by the global society and continues to be a world concern. Pollution caused by toxic metals receives considerable attention due to the impacts they introduce to the environment and human health. Metals such as mercury (Hg), cadmium (Cd), arsenic (As), lead (Pb), nickel (Ni), chromium (Cr) and cobalt (Co) are highly toxic even in minor quantity. Recently contaminations of metals in food stuffs and associated health risks have been reported extensively and led interest of searching for medicines of metals toxicity. In this regard, dietary components can be appropriate option and offer great potentials of being therapeutics against metals, with least side effects. In this study, essential micronutrients selenium (Se) and zinc (Zn), dietary supplement dihydrolipoic acid (DHLA) and food additive butylated hydroxytoluene (BHT) have been used to evaluate the protective actions against metals-induced harmful effects in PC12 cells, which is a model cell line for molecular toxicity assessment. In this research it was hypothesized that “food components have regulatory effects on metals mediated toxicity at cellular level”.

In chapter 2, Se was used to detoxify Cd in PC12 cells in a concurrent exposure. It was hypothesized that Se has a protective role on Cd-induced cytotoxicity. The purpose was to investigate the mechanism/s of cyto-protection by Se (Se^{4+}) against Cd (Cd^{2+})-induced cytotoxicity. Additionally, Se (5, 10, 20 and 40 μM) and Cd (2.5, 5 and 10 μM)-induced cytotoxicity was determined. In this research certain cytotoxicity assessment techniques were conducted such as cell viability assay, lactate dehydrogenase activity assay, GSH measurement by DTNB, DNA fragmentation determination, and western blotting protein expression assay. Cytotoxicity assays

and western blot analyses confirmed that Se (≥10 µM) promotes autophagic cell death *via* inhibition of mTOR activation and p62 accumulation due to increase of cellular oxidative stress. On the other hand, co-presence of non-toxic Se (5 µM) and toxic Cd (5 µM) was shown to increase cell viability, glutathione and glutathione peroxidase 1 (GPx1) levels, and to decrease DNA fragmentation and lactate dehydrogenase activity compared to Cd-treated (5 µM) cells alone. Furthermore, western blot analyses of cytochrome c and ERK1 indicated that Cd-induced apoptotic cell death in PC12 cells. However, the co-exposure of Se (5 µM) with Cd (5 µM) significantly decreases the release of cytochrome c into cytosol from mitochondria, and up-regulates ERK1 protein to inhibit Cd (5 µM)-induced apoptosis. In conclusion, Se (≥10 µM) possess cytotoxicity in PC12 cells; however, co-presence of Se (5 µM) with Cd (5 µM) protects against Cd-induced apoptosis in PC12 cells due to inhibition of Cd-induced oxidative stress and subsequently suppression of mitochondrial apoptosis pathway (Fig. 1).

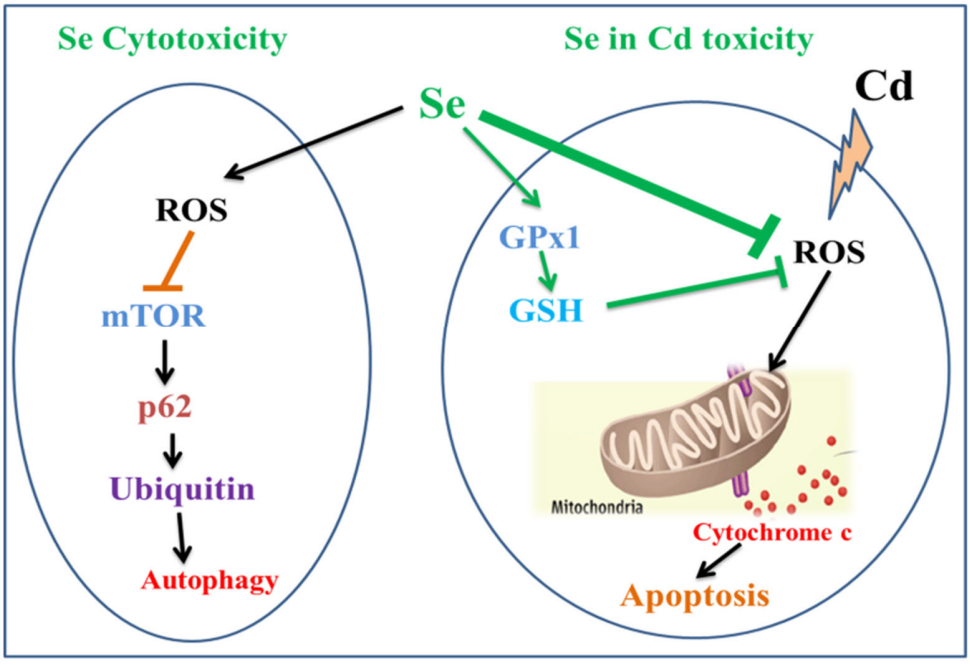

Figure 1: Biomolecular mechanisms of Se toxicity, and Se-protection against Cd-mediated toxicity in PC12 cells.

Another extremely dangerous environmental contaminant is Hg, which causes human diseases including neurological disorders. DHLA is a potent antioxidant that acts through multiple

mechanisms. In chapter 3, it was hypothesized that DHLA has a protective role on iHg-induced cytotoxicity. The objectives of this study were to investigate mechanism/s of toxicity of iHg, along with the amelioration of DHLA against iHg-induced toxicity on PC12 cells. In this research to assess the above mentioned hypothesis several cytotoxicity investigation methods were conducted such as cell viability analysis by trypan blue, DNA fragmentation analysis by agarose gel electrophoresis, lactate dehydrogenase assay, glutathione measurement by DTNB, western blotting analysis for protein expression, apoptosis detection by flow cytometry and inorganic mercury accumulation by reduction vapor AAS. The cells were treated with $HgCl_2$ (Hg^{2+}) (0-2.5 μM) for 48 h which showed significant toxic effects. iHg resulted in cell viability decrease and lactate dehydrogenase release from the cells. iHg also caused DNA fragmentation, cellular glutathione reduction and accumulation Hg. Additionally, expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2 and HO1 in cells which are pro-surviving proteins were downregulated. Moreover, cleaved caspase 3 and cytochrome that are pro-apoptotic proteins were upregulated after Hg^{2+} (2.5 μM) treatment and thus#confirming#apoptosis induction. However, pretreatment with DHLA (50 μM) 3#h before Hg^{2+} (2.5 μM)-treatment displayed inhibition against iHg-induced cytotoxicity. DHLA (50 μM)-pretreatment reversed cell viability loss and LDH release induced by iHg-toxicity. DHLA (50 μM)-pretreatment reduced DNA damage, GSH decrease and inhibiting Hg accumulation caused by iHg as well. Moreover, DHLA (50 μM) pretreatment inverted the expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2 and HO1 pro-survival proteins, indicating cellular survival. In addition, cleaved caspase 3 and cytochrome c pro-apoptotic proteins were downregulated. Further flow cytometry method showed reduced apoptotic cells in DHLA (50 μM)-pretreated group. So, it was suggested that DHLA could inhibit iHg-cytotoxicity *via* strengthening antioxidant defense, and inhibiting Hg accumulation and apoptosis (Fig. 2).

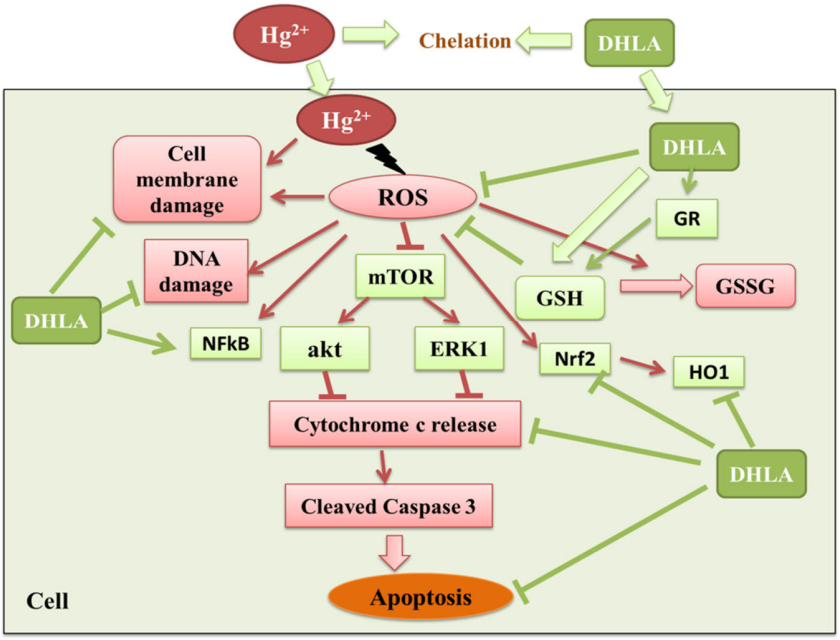

Figure 2: Biomolecular mechanisms of Hg toxicity, and DHLA-protection against Hg-mediated toxicity in PC12 cells.

In chapter 4, the Se-protection against iHg-mediated toxicity in PC12 cells was investigated in simultaneous exposure. The purposes of this study were to investigate the inhibition of iHg-induced toxicity by Se-co-exposure and its molecular mechanisms. In this research to assess the above mentioned hypothesis several cytotoxicity investigation methods were conducted such as cell viability analysis by trypan blue, DNA fragmentation analysis by agarose gel electrophoresis, lactate dehydrogenase assay, glutathione measurement by DTNB, western blotting analysis for protein expression, apoptosis detection by flow cytometry. The cells were treated with $HgCl_2$ (Hg^{2+}) (0-5 µM) for 48 h which showed significant toxic effects. Cytotoxicity assays showed that iHg (5 µM) encouraged cell viability loss, cell membrane integrity degradation, glutathione oxidation, DNA damage, oxidative stress and intrinsic apoptosis induction. In addition, iHg (5 µM) stimulated down-regulation of pro-survival proteins, e.g., mTOR, p-mTOR, akt and ERK1, and up-regulation of pro-apoptotic proteins, e.g., cleaved caspase 3 and cytochrome c in PC12 cells after 48 h of incubation.

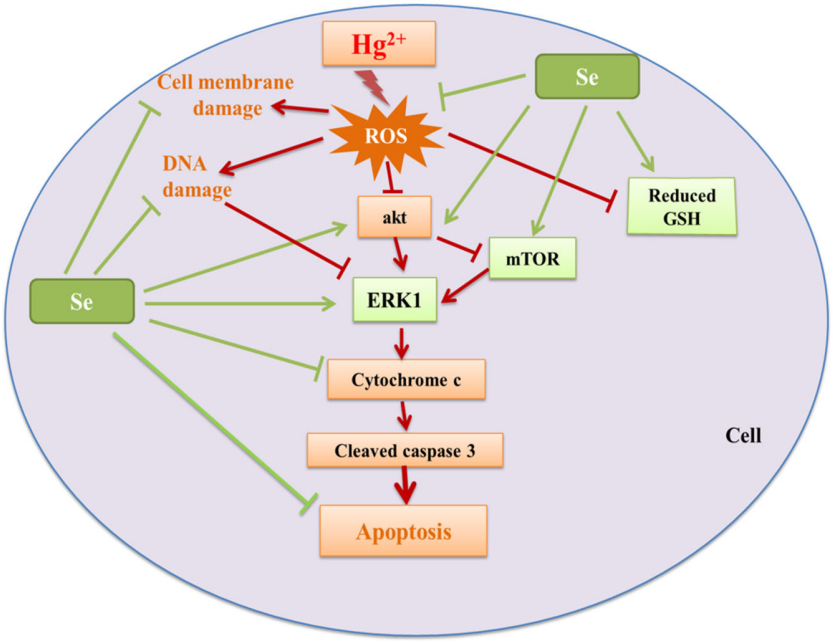

Figure 3: Biomolecular mechanisms of Se-protection against Hg-mediated toxicity in PC12 cells.

On contrary, co-treatment of Se (5 µM) seemed to prevent intrinsic apoptosis and oxidative stress induced by iHg (5 µM) through improving GPx1 contents, glutathione contents, DNA degradation, cell membrane integrity. Moreover, co-treatment of Se (5 µM) with iHg (5 µM) increased mTOR, p-mTOR, akt, ERK1 and caspase 3, and decreased cleaved caspase 3 and cytochrome c, indicating inhibition of apoptosis. The role of Nrf2 and HO1 activation was insignificant in iHg (5 µM)-toxicity or Se (5 µM)-protection mechanisms in this cell line. These findings suggested that glutathione oxidation plays crucial role in iHg induced oxidative damages and Se co-treatment reduces iHg-cytotoxicity through its antioxidant activities (Fig. 3).

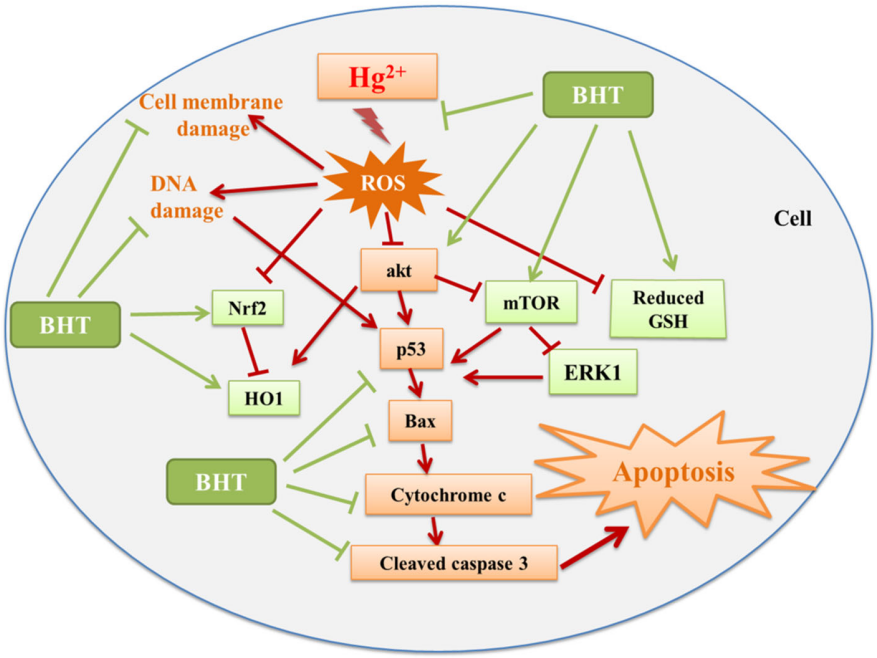

Figure 4: Biomolecular mechanisms of BHT-protection against Hg-mediated toxicity in PC12 cells.

BHT is a phenolic component largely used as a food additive because of its antioxidant properties. However, the molecular mechanisms of BHT-induced antioxidant properties against heavy metals-influenced toxicity are little investigated. In chapter 5, the inhibitory action of BHT was assessed against iHg-induced cytotoxicity by a simultaneous exposure in PC12 cells. In this chapter it was hypothesized that BHT has a modulating role on iHg-induced cytotoxicity. The purpose of this study was to evaluate the ameliorative effects of BHT against iHg-toxicity in PC12 cells. PC12 cells were incubated with/without $HgCl_2$ (Hg^{2+}) (5 µM) and BHT (100 µM) for 48 h and explored further. In this research to assess the above mentioned hypothesis several cytotoxicity investigation methods were conducted such as cell viability analysis by trypan blue, DNA fragmentation analysis by agarose gel electrophoresis, lactate dehydrogenase assay, glutathione measurement by DTNB, western blotting analysis for protein expression, apoptosis detection by flow cytometry. Cells treated by iHg (5 µM) caused a significant cell viability loss and cell membrane damage. iHg (5 µM)-incubated cells caused a significant glutathione decrease, DNA damage as well. Moreover, iHg (5 µM)-treatment resulted in downregulated expressions of p-akt, akt, mTOR, ERK1, Nrf2, HO1, Bcl-xL, caspase 7 and caspase 3 proteins; and upregulated expressions of p53, Bax,

cytochrome c and active caspase 3 proteins. However, the simultaneous exposure of BHT (100 μM) and iHg (5 μM) exhibited ameliorative effects against iHg-influenced toxicity. Simultaneous exposure improved cell viability and LDH release. In addition, the simultaneous exposure of BHT (100 μM) and iHg (5 μM) declined DNA damage and GSH oxidation as well. Moreover, BHT (100 μM) co-treatment successfully reversed the akt, p-akt, mTOR, ERK1, Nrf2, HO1, caspase 7, caspase 3, cleaved caspase 3 and cytochrome c protein expressions. BHT (100 μM) in co-exposure upregulated the pro-survival proteins akt, p-akt, mTOR, ERK1, Nrf2, HO1; and downregulated cleaved caspase 3 and cytochrome c protein expressions. These outcomes suggested that BHT attenuates iHg-influenced cytotoxicity *via* boosting antioxidant defense, and inhibiting cytotoxicity and intrinsic pathway of apoptosis (Fig. 4).

In chapter 6, the effect of Zn on inorganic Hg-induced cytotoxicity in the PC12 cells were investigated. Zinc (Zn) is an essential micronutrient with a wide range of antioxidant properties. The PC12 cells were incubated with $HgCl_2$ (5 μM) for 48h with/without 1 h prior $ZnCl_2$-treatment (100 μM) and further analysis were executed. In this research several cytotoxicity investigation methods were used such as cell viability analysis by trypan blue, DNA fragmentation analysis by agarose gel electrophoresis, lactate dehydrogenase assay, glutathione measurement by DTNB method, western blotting analysis for protein expression and apoptosis detection by flow cytometry. The cells incubated with only iHg (5 μM) revealed cell viability loss and cell membrane degradation. Cells treated by iHg (5 μM) also showed intensified DNA damage, glutathione level reduction, and severely augmented apoptosis. Consequently, iHg (5 μM)-treated cells depicted a significant downregulating expressions of akt, mTOR, ERK1, Nrf2, HO1, Bcl-2, Bcl-xL pro-survival proteins; and upregulating expressions of p53, Bax, cytochrome c and cleaved caspase 3 pro-apoptotic proteins, indicating intensive apoptosis induction *via* mitochondrial/intrinsic pathway. On the other hand, PC12 cells pre-incubated with Zn (100 μM) before iHg (5 μM)-treatment demonstrated a significant improvement in cell viability and cell membrane. In addition, pre-treatment of Zn showed reduced DNA damage and increased glutathione amount. Flow cytometry study confirmed that pre-treatment of Zn showed a significant improvement in apoptotic cell number. Pre-treatment of Zn before iHg-exposure, influenced in the protein expressions of the PC12 cells. A significant upregulation in mTOR, akt, p-akt, ERK1, Nrf2, HO1, Bcl-2 and Bcl-xL pro-survival proteins, and downregulation in p53, Bax, cytochrome c and cleaved caspase 3 pro-

apoptotic proteins were observed, which indicates inhibition of intrinsic/mitochondrial apoptosis by Zn-pretreatment. The findings suggested that Zn-pretreatment inhibits iHg-induced cytotoxicity and intrinsic apoptosis, not only *via* improving reduced glutathione amount but also encouraging Nrf2-HO1 pathway activation (Fig. 5).

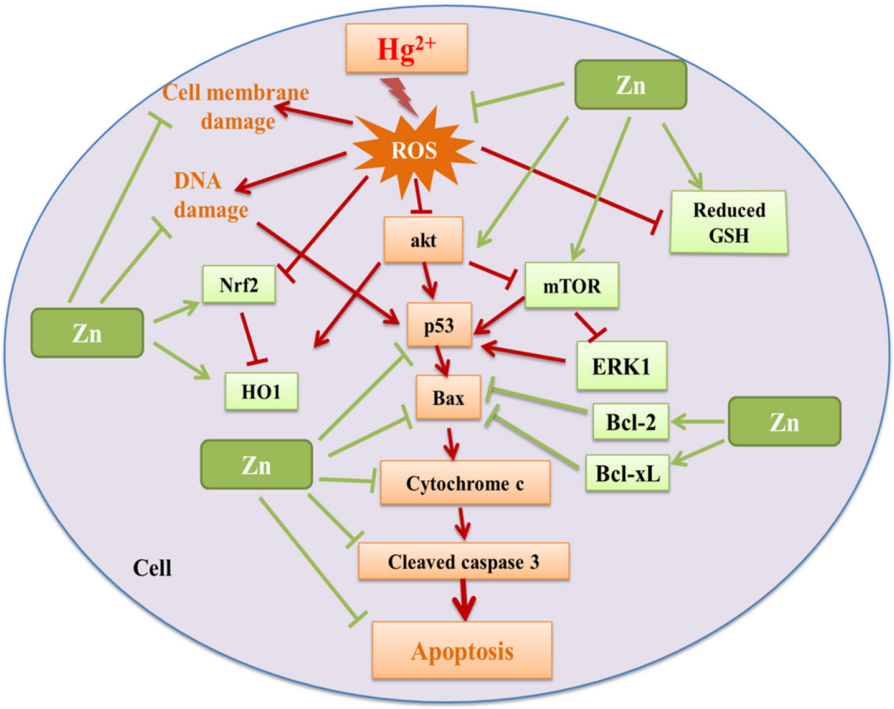

Figure 5: Biomolecular mechanisms of Zn-protection against Hg-mediated toxicity in PC12 cells.

Heavy metals (Cd and Hg)-exposure even in trace amount can cause deleterious health effects and diseases in human. However, these diet components (Se, DHLA, BHT and Zn) have proper ameliorative actions against metal induced toxicity. These diet components improved the cellular condition *via* hindering oxidative stress and strengthening antioxidant defense mechanisms. Moreover, these diet components ameliorated the cell-membrane integrity, intact DNA, reduced glutathione content and cell viability; and protected from metal induced ROS generation, oxidative stress and apoptosis. This research recommended that Se, DHLA, BHT and Zn could be used as therapeutic agents in combating metal induced effects in biological systems and suggested further study in animal models for better understanding.