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Signaling and Proteomics in Methylmercury Exposure

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Methylmercury (MeHg; CH_3HgCl) is, second only to cadmium as being, the most toxic on the earth. Inorganic mercury from various waste sources can be easily methylated by bacteria in water and subsequently ingested by fishes and then highly accumulated in human. Although toxicity from mercury exposure occurs with both organic and inorganic forms, organic mercury is more potently toxic to central nervous system. Minamata disease is an example of organic mercury toxicity.

Mercury's effect on the biochemical level have been studied extensively including DNA damage, alteration of protein structure, alteration of the transport of calcium through calcium channels leading to the disruption of the communication between cells, induction of free radical formation and inhibition of the antioxidant enzyme glutathione peroxidase. Thus far, MeHg has been suggested to exert its toxicity through its high reactivity to thiols of bioactive proteins, elevation in intracellular Ca^{2+} concentration, and generation of reactive oxygen species, but its mechanism remains poorly understood.

In this study we used conventional biochemical method for investigating a relationship between various cytotoxic mediators (PLA_2, PC-PLC, SMase and Ca^{2+}) in MDCK cells and utilized display proteomics to identify key proteins in methylmercury toxicity in human neuroblastoma SH-SY5Y cells.

First, phospholipase A_2 (PLA_2) has been implicated in various forms of cell injury. This enzyme produces lipid-derived bioactive chemical mediators such as arachidonic acid (AA), prostaglandins, leukotrienes, platelet-activating factor, and lysophospholipids. In the oxidation process of AA, ROS is also generated. Among various types of mammalian PLA_2, group IV cytosolic PLA_2 ($cPLA_2$) may be of particular importance as a mediator of cellular injury. This enzyme, which has a high specificity for AA at the *sn*-2 position of phospholipids, is activated by elevation in $[Ca^{2+}]_i$ and ROS. The $cPLA_2$ is activated through its translocation to membranes by $[Ca^{2+}]_i$ elevated during stimulation and phosphorylation by mitogen-activated protein (MAP) kinase in response to various environmental stresses and hormones.

Verity *et al.* suggested a role for PLA_2 in MeHg-induced cell injury when they reported that this toxicant-induced cytotoxicity with a concomitant release of AA in cerebellar granule cells.

However, the precise mechanism by which MeHg activates this neuronal type of PLA_2 and the correlation between the activation of this enzyme and the cytotoxicity remain to be fully defined. Moreover, the presence of $cPLA_2$ in neuronal cells is still controversial. These findings formed the basis of this study, i.e., whether MeHg can activate $cPLA_2$ in these kidney cells and whether the activation can lead to MeHg-induced cytotoxicity.

Second, sphingomyelinase (SMase) catalyzes the hydrolysis of membrane sphingomyelin to generate ceramide and phosphocholine. Generation of the membrane sphingolipid ceramide may play a crucial role in cell response such as cell differentiation, cell cycle arrest, cellular senescence, and programmed cell death or apoptosis. There are several isoforms of SMase, distinguished by different pH optima. Neutral Sphingomyelinase (N-SMase) prefers pH of 7.0 to 7.5, whereas Acid Sphingomyelinase (A-SMase) requires pH 4.5 to 5.5 and DAG for activation. It has been previously reported that A-SMase, which is defective in patients with Niemann-Pick disease, associated with the lysosomal intracellular compartment or the caveolae.

Third, phosphatidylcholine–specific Phospholipase C (PC-PLC) is known to couple to SMase, a praticularly acidic form (A-SMase) and to be required in oxidative glutamate toxicity of neuronal cells. Moreover, it has known that A-SMase is laid downstream of PC-PLC in surface receptor-ligand interaction such as TNF-α and Fas signaling.

Finally, proteomics is the study of biology by use of proteome analysis technologies. It combines high-resolution two-dimensional gel electrophoresis, high-sensitivity mass spectrometry, and continuously expanding protein databases. Two-dimensional gel electrophoresis (2-DE) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or others. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained.

Here we show that MeHg induced AA release by activating $cPLA_2$ through multiple mechanisms including calcium, phosphorylation and oxidative stress. $AACOCF_3$, a specific inhibitor of $cPLA_2$, blocked MeHg-induced AA release and intracellular ROS generation, but not LDH release. N-acetyl cysteine, an antioxidant, could not protect against the cytotoxicity of MeHg despite a significant inhibition of the AA release.

On the other hand, MeHg induced a slight increase in DAG production and ceramide generation with concomitant hydrolysis of SM. MeHg markedly activates the activity of A-SMase, not N-SMase. Monensin and NH_4Cl, indirect inhibitors of A-SMase inhibited

ceramide generation but not LDH release. Inhibition of PC-PLC, a well-known upstream activator of A-SMase, inhibited the MeHg-induced DAG generation, A-SMase activation, ceramide generation, and LDH release.

MeHg increased intracellular calcium in a bimodal pattern with a sharp peak at 1 min and sustained increase up to 10 min. Chelating of extracellular calcium partially attenuated a short-term cytotoxicity of MeHg with the abolishment of the sharp peak at 1 min and significant reduction in the sustained Ca^{2+} increase. Interestingly, D609, PC-PLC inhibitor, completely decrease not only MeHg-induced calcium increase but also LDH release. This suggests that MeHg-induced response is composed of PC-PLC mediated Ca^{2+} mobilization component and a Ca^{2+} influx component, with the influx component being dependent on mobilization component and therefore relating with cell death.

Taken together, the present study indicates that MeHg activates $cPLA_2$ through Ca^{2+}-dependent and oxidative pathways. However, the resulting production of AA and ROS may not be implicated in its cytotoxicity, rather PC-PLC pathway is likely to play an important role in the cytotoxicity by MeHg through $[Ca^{2+}]_i$ increase by the Ca^{2+} mobilization and influx.

On the other hand, to examine the change of protein level after exposure to MeHg, total proteins from SH-SH5Y cells were extracted and separated by 2-DE and detected by silver staining. Several spots in MeHg-treated cells showed different profiles compared with untreated cells and were analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Further comparative studies to identify, quantity, and characterize the proteins differentially expressed in MeHg-treated SH-SY5Y cells will give insight into biomolecular mechanisms of MeHg-induced toxicity.