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Methylmercury Induces Cytotoxicity through Inhibition of PTEN Activity by a Decrease in Its Solubility

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Methylmercury is a major environmental pollutant that exhibits neurotoxicity. We previously reported that proteolytic systems such as the ubiquitin–proteasome system (UPS) and autophagy are involved in methylmercury toxicity. It is known that the intercellular level of PTEN, a phosphatase involved in autophagy inhibition, is regulated by the UPS. In this study, we coincidentally found that the PTEN level was decreased by methylmercury in the lysate solubilized with RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS). However, the decrease in PTEN level caused by methylmercury was scarcely observed in RIPA buffer containing 2% SDS. These results suggest that methylmercury lowers the solubility of PTEN protein. Moreover, phosphorylation of Akt, a protein kinase that is negatively controlled via PTEN, was accelerated in accordance with a decrease in PTEN protein solubility. This suggests that methylmercury may inhibit PTEN activity by decreasing its solubility. Moreover, PTEN overexpression confers resistance to methylmercury in SH-SY5Y neuroblastoma cells. These results suggest that PTEN is a novel factor involved in reducing methylmercury toxicity and that methylmercury inhibits PTEN activity by decreasing the solubility of PTEN protein, thereby increasing cytotoxicity.

Key words methylmercury, PTEN, cytotoxicity, insolubility

Methylmercury is a representative environmental pollutant that causes central nervous system (CNS) damage.[1,2] Mercury in the natural world is transformed by bacterial action to methylmercury and accumulates along the food chain so that it is found at relatively high levels in large fish. In recent years, it has become a global problem, confirmed by CNS defects in babies born to mothers who consume large amounts of fish.[3,4] Nevertheless, the mechanisms involved in methylmercury toxicity and the protection against it are mostly still unknown.

We have found that the ubiquitin–proteasome system (UPS, a proteolytic system) and autophagy are involved in methylmercury toxicity.[5-7] We have also reported that excessively induced autophagy may be involved in enhancing methylmercury toxicity.[7] Chang *et al*. reported that methylmercury reduces the levels of mammalian target of rapamycin (mTOR, an autophagy inhibitor), thereby inducing autophagy.[8] This results in the inhibition of differentiation of neural stem cells into neurons. In addition, it has been suggested that in astrocytes and murine embryonic fibroblasts, autophagy may be a defense mechanism in response to methylmercury toxicity.[9,10]

Autophagy is a system for degrading proteins in cells, serving to prevent the accumulation of hypersynthesized proteins and abnormal proteins generated by a variety of stresses.[11] Autophagy also contributes to biological homeostasis, including in the performance of protein cycles during starvation states.[12] It is known that autophagy is induced by the activation of AMP-activated protein kinase when cells experience hypoglycemic or hypoxic starvation states.[13] It is further known that when there is recovery to a normal intracellular nutrition state, mTOR is activated, and autophagy induction is inhibited.[14] mTOR is a serine–threonine kinase activated by protein kinase B (Akt).[15] Moreover, it is known that Akt is activated via phosphatidylinositol-3,4,5-trisphosphate (PIP3), phosphorylated from phosphatidylinositol-4,5-bisphosphate (PIP2) by phosphoinositide 3-kinase (PI3K); this signal-transduction system is known as the PI3K/Akt/mTOR pathway.[16] This pathway is known to be negatively controlled by a phosphatase called PTEN (phosphatase and tensin homolog deleted on chromosome 10).[16] PTEN exists mainly in the cytoplasm. When PTEN moves to the cell membrane, by dephosphorylating PIP3 to PIP2, it inhibits Akt activation.[17] In recent years, many reports have emerged that PTEN functional abnormalities resulting in hyperactivation of Akt and causing progression of cancer.[18-20] This suggests the involvement of autophagy inhibition mediated by Akt hyperactivation.[21] Intracellular levels of PTEN are regulated by the UPS. Involved in this process, as a ubiquitin ligase, is a neural precursor cell-expressed developmentally down-regulated protein 4-1 (NEDD4-1).[22] It is reported that NEDD4-1 levels increase by oxidative stress, and then ubiquitination of PTEN is pro-

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moted.[23] Meanwhile, antioxidant treatment has been reported to inhibit autophagy inducement by methylmercury,[10] suggesting that oxidative stress may be involved in autophagy inducement by methylmercury. As stated above, PTEN is a major factor in autophagy regulation, and it is known that its activity is controlled by various stresses.[24,25] The role of PTEN in methylmercury toxicity, however, is completely unknown. In the present study, SH-SY5Y neuroblastoma cells were used to investigate the relationship between methylmercury toxicity and PTEN.

MATERIALS AND METHODS

Cell Culture and Cell Viability SH-SY5Y cells (derived from human neuroblastoma) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (Bio-West, Nuaille, France), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin in a 5% humidified CO_2 (95%) atmosphere at 37°C. Before the experiments, the cells were seeded (1×10^6 cells/well) in a 6-well plate for immunoblotting or quantitative polymerase chain reaction (PCR), or in a 96-well plate (5×10^3 cells/well) for cell viability, and incubated for 24 h.

Cell viability was measured by incubating the cells with alamarBlue® (Biosource, Camarillo, CA, USA) according to the manufacturer's protocol. Briefly, cells were incubated with 10% alamarBlue® solution for 2 h, and the resulting fluorescence was measured at an excitation of 544 nm and emission of 590 nm using a Gemini XPS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

Fractionation of Soluble and Insoluble Proteins and Immunoblotting Cells were harvested in RIPA buffer [1 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid] supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation (20,000 *g*, 4°C for 15 min), supernatants were collected as a 0.1% SDS soluble fraction. After that, the remaining pellet was dissolved in the same buffer containing 2% SDS, and centrifuged again (20,000 *g*, 4°C for 15 min). The supernatant was used as 0.1% SDS insoluble fraction.

The samples were subjected to SDS-polyacrylamide gel electrophoresis. The obtained gel was transferred to an Immobilon-P membrane (Millipore, Burlington, MA, USA) and stained with antibodies against PTEN (Everest Biotech, Oxfordshire, UK), Actin (Santa Cruz Biotechnology, Dallas, TX, USA), Akt (Cell Signaling Technology, Danvers, MA, USA), phospho (T308)-Akt (Cell Signaling Technology), and V5 (Fujifilm-Wako Pure Chemical, Osaka, Japan).

Measurement of PTEN mRNA by Real-Time Quantitative PCR Total RNA was purified from SH-SY5Y cells with Isogen II (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Obtained RNA was reverse-transferred to cDNA with a PrimeScript® RT reagent kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Quantitative PCR was performed with SYBR Premix EX Taq (Takara), a Thermal Cycler Dice® (Takara), and the following primers: PTEN, 5'- CGACGGGAAGACAAGTTCAT-3' (sense) and 5'- AGGTTTCCTCTGGTCCTGGT-3' (antisense); GAPDH 5'- GCACCGTCAGGCTGAGAAC-3' (sense) and 5'- TGGTGAAGACGCCAGTGGA-3' (antisense). PTEN mRNA levels were normalized to GAPDH mRNA.

Establishment of PTEN-V5 Overexpressed SH-SY5Y Cells The coding region of PTEN gene was amplified with the following primers: 5'-GGGAAGGTACCATGACAGCCATCATCAAAGAG-3' (sense) and 5'-TTTTTGCGGCCGCCGACTTTTGTAATTTGTGTATGCT-3' (antisense). The resulting PCR product was inserted into pEF5V/FRT/V5-DEST vector by using restriction enzymes Not1 and Kpn1 (Takara). The purified vector was transfected to SH-SY5Y cells for 48 h by Screenfect (Fujifilm-Wako Pure Chemical) according to the manufacture's protocol and the non-transfected cells were removed by addition of hygromycin B (150 µg/mL) to the culture media. Survived cells were grown and used as PTEN-V5 overexpressed SH-SY5Y cells.

RESULTS

Methylmercury Lowers the Solubility of PTEN Protein It is known that the intercellular level of PTEN is regulated by the UPS.[22] First, to investigate effects of methylmercury on PTEN protein levels, immunoblotting was performed for recovered cells after their solubilization in RIPA buffer containing 0.1% SDS. The result was that methylmercury decreased PTEN protein levels in a dose-dependent manner (Fig. 1A), while PTEN mRNA levels were mostly unaffected by methylmercury (Fig. 1B). It is noteworthy that almost no cytotoxicity was confirmed when methylmercury was of a concentration at which a decrease of PTEN protein was observed (Fig. 1C). These results suggest that the decrease of PTEN protein levels may be caused by methylmercury prior to the occurrence of cytotoxicity, and that methylmercury may cause a decrease in PTEN protein levels by accelerating its degradation. Even after pretreatment with a proteasome inhibitor, the same PTEN protein level decreases, caused by methylmercury, were confirmed (data not shown); the same results were obtained even after pretreatment with a lysosome inhibitor (data not shown). These results suggest that a proteolytic system may not be involved in the decrease of PTEN protein levels by methylmercury.

Meanwhile, when SH-SY5Y cells treated with methylmercury were solubilized in RIPA buffer containing 2% SDS, there was almost no decrease in PTEN protein levels caused by methylmercury (data not shown). Then, to investigate the effects of methylmercury on PTEN solubility, the remaining pellet that could not be solubilized in the 0.1% SDS RIPA buffer was resolubilized in a 2% SDS RIPA buffer. The obtained lysates were used as 0.1% SDS insoluble fractions, and an investigation was made of PTEN protein levels in both fractions. As a result, accompanying the decrease in PTEN protein levels in 0.1% SDS soluble fractions were increases in PTEN protein levels in 0.1% SDS insoluble fractions (Fig. 2). These results showed that a decrease of PTEN protein solubility was involved in the decrease of PTEN protein level caused by methylmercury in 0.1% SDS soluble fractions.

Akt Is Phosphorylated by Decrease of PTEN Protein Solubility Caused by Methylmercury It is hypothesized that PTEN activity will be affected by its decrease in solubility caused by methylmercury. PI3K is negatively controlled through the dephosphorylation of PIP3 to PIP2 by PTEN. Meanwhile, Akt, a downstream factor of PI3K, is activated via phosphorylation of Thr308.[26] We thus investigated the effect over time of methylmercury on Akt phosphorylation. The result was that Akt phosphorylation was accelerated in

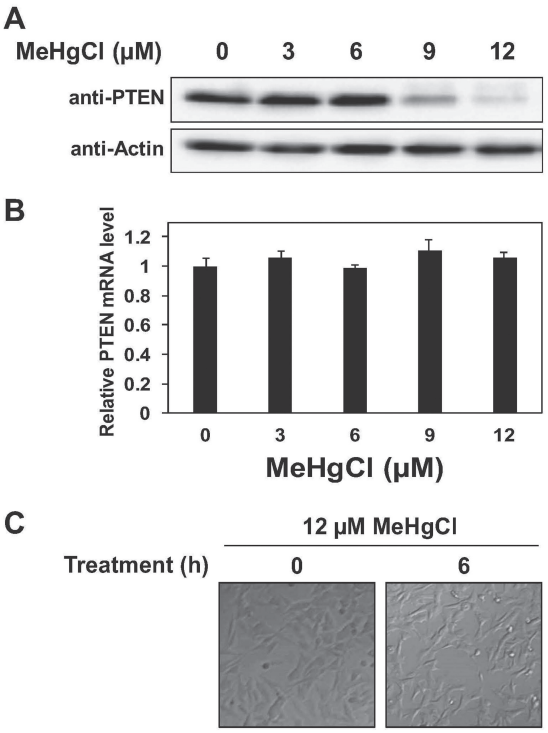

Fig. 1. Effects of Methylmercury on the Expression of PTEN in SH-SY5Y Cells

SH-SY5Y cells were seeded on a 6-well plate and treated with indicated concentration of methylmercuric chloride (MeHgCl) for 6 h. The level of PTEN protein was measured by immunoblotting (A), and the level of PTEN mRNA was determined by qPCR (B). Representative image of the cells treated with MeHgCl (12 μM) for 6 h were monitored by Leica DM IRB microscope (C). Values represent mean ± SD of three individual experiments.

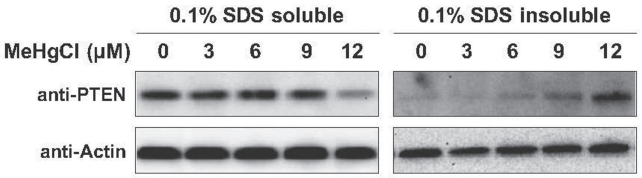

Fig. 2. Effects of Methylmercury on the Solubility of PTEN in SH-SY5Y Cells

SH-SY5Y cells were seeded on a 6-well plate and exposed to indicated concentration of methylmercuric chloride (MeHgCl) for 6 h. The cells were lysed with RIPA buffer containing 0.1% SDS (0.1% SDS soluble fraction). Remaining pellet was redissolved into RIPA buffer containing 2% SDS and used as 0.1% SDS insoluble fraction. PTEN level in each fraction was measured by immunoblotting. Actin was used as loading control.

accordance with the PTEN protein increase in the 0.1% SDS insoluble fraction (Fig. 3). Meanwhile, even when cells overexpressing PTEN were treated with methylmercury for 6 h, considerable PTEN protein still existed in 0.1% SDS soluble fractions, and almost no phosphorylation of Akt caused by methylmercury under these conditions (data not shown). These results suggest that a decrease in PTEN protein solubility may be related to the phosphorylation of Akt by methylmercury.

PTEN Overexpression Reduces Cytotoxicity Caused by Methylmercury The results shown in Fig. 3 suggest that methylmercury inhibits PTEN activity by decreasing its solubility; we considered that this phenomenon may have an impact on cytotoxicity caused by methylmercury. We thus investigated the effects of PTEN overexpression on methylmercury sensitivity of SH-SY5Y cells. Cells that stably over-

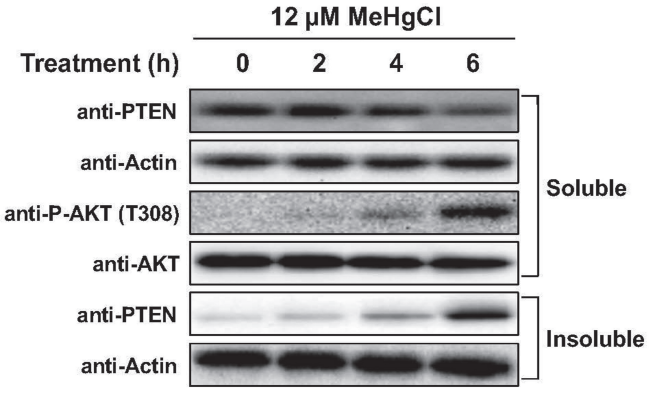

Fig. 3. Effects of Methylmercury on Phosphorylation of Akt in SH-SY5Y Cells

SH-SY5Y cells were seeded on a 6-well plate and exposed to 12 μM methylmercuric chloride (MeHgCl) for indicated time. The level of PTEN, Akt, and phospho (T308)-Akt in 0.1% SDS soluble fraction (Soluble) were measured by immunoblotting (upper). Remaining pellet was redissolved into RIPA buffer containing 2% SDS (Insoluble), and PTEN level was measured. Actin was used as loading control.

expressed PTEN fused with a V5 tag at its C terminal (PTEN-V5) showed greater methylmercury resistance than control cells (Fig. 4A). Fig. 4B shows confirmation of PTEN-V5 expression via immunoblotting. These results suggest that PTEN is a methylmercury toxicity reducing factor and that cytotoxicity increases as the result of PTEN activity inhibition by methylmercury.

DISCUSSION

The present study suggests that PTEN is a novel factor involved in reducing methylmercury toxicity and that methylmercury inhibits PTEN activity by lowering the solubility of PTEN protein, thereby increasing cytotoxicity. There is no report that insolubilization of PTEN protein triggers its reduced activity, and this finding suggests the existence of a novel mechanism involved in the inhibition of PTEN activity.

Many research groups, including ours, have reported that methylmercury induces autophagy.[7-10] The PI3K/Akt pathway that is negatively controlled via PTEN is involved in the activation of mTOR, an autophagy inhibitor.[21] The results shown in Fig. 3 suggest that methylmercury may inhibit PTEN activity by decreasing its solubility. Nevertheless, even under conditions where it has been confirmed that PTEN activity is inhibited by methylmercury, autophagy induction potency by methylmercury was maintained as before (data not shown). This suggests that the inhibition of the PI3K/Akt/mTOR pathway is not involved in autophagy induction by methylmercury in SH-SY5Y cells. We also found that methylmercury inhibits the phosphorylation of AMPK involved in autophagy induction (unpublished data). Therefore, it is possible that unknown pathways other than mTOR and AMPK are involved in the induction of autophagy by methylmercury.

PI3K, activated by many survival factors, phosphorylates Akt via 3-phosphoinositide-dependent protein kinase-1 (PDK1).[26] Akt then phosphorylates the pro-apoptotic proteins (Bad, Bax, etc.), thereby inhibiting apoptosis.[27,28] Meanwhile, PTEN inhibits the PI3K/Akt pathway, thereby inducing apoptosis.[29] It is also known that methylmercury induces apoptosis within neuronal cells, causing CNS damage.[30,31] Yet because PTEN overexpression inhibited cytotoxicity by methylmer-

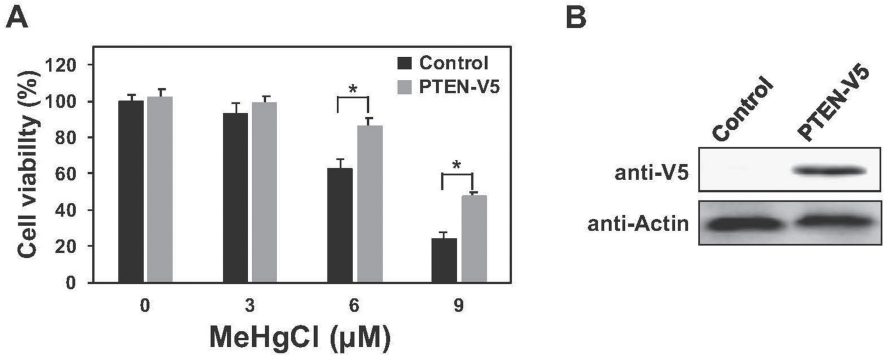

Fig. 4. Effects of Overexpression of PTEN on Methylmercury-Induced Cytotoxicity

Empty vector transfected-SH-SY5Y cells (Control) or PTEN-V5 transfected-SH-SY5Y cells (PTEN-V5) were exposed to methylmercuric chloride (MeHgCl) for 24 h, and the cell survival was measured by alamarBlue® assay (A). Expression of PTEN-V5 was detected by immunoblotting (B). Values represent mean ± SD of three individual experiments. *$P < 0.05$ vs control as indicated by *t*-test. Actin was used as loading control.

cury, at the least, within SH-SY5Y cells, the PI3K/Akt pathway inhibition effects shown by PTEN suggest that this pathway is not involved in reducing methylmercury toxicity.

It is known that Cys71 and Cys124 within PTEN are oxidized by hydrogen peroxide, and its activity is reduced by the formation of disulfide bonds.[32] Methylmercury has been shown to cause neuronal cell death by inducing oxidative stress.[33,34] It is thought possible that PTEN is oxidized by methylmercury and is thus insolubilized. However, even in a mutated PTEN with a substituted Cys124, a decrease in solubility to a similar extent as wild-type PTEN was confirmed (data not shown). This suggests that the formation of disulfide bonds between Cys71 and Cys124 within PTEN may not be involved in the decrease of PTEN protein solubility by methylmercury. A recent report states that PTEN is modified by methylmercury;[35] it may be that the binding of methylmercury to PTEN cysteine residues causes the decrease in its solubility. In the future, we plan to conduct a detailed investigation of relationships between cysteine residues and PTEN solubility with the aim of clarifying the mechanisms involved in inhibition of PTEN activity by methylmercury.

PTEN has been identified as a tumor suppressor.[18] It is one type of protein tyrosine phosphatase (PTPase), and it has been reported to be involved in cell proliferation and neuron growth during development.[36] A recent report states that PTEN is involved in adjustments to synaptic plasticity in the brain.[37] PTEN functional changes within synapses may be linked with imperfect synaptic functions, accompanied by behavioral and cognitive ramifications. In other words, it is possible that PTEN plays an important role in CNS damage by methylmercury. In the future, we will identify the substrate of PTEN involved in methylmercury toxicity, which should allow the mechanisms related to reduction of methylmercury toxicity via PTEN to be clarified.

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Conflict of Interest The authors declare no conflict of interest.

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