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Superoxide produced in the matrix of mitochondria enhances methylmercury toxicity in human neuroblastoma cells

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ABSTRACT

The mechanism of intracellular metabolism of methylmercury (MeHg) is not fully known. It has been shown that superoxide ($O_2^{\bullet -}$), the proximal reactive oxygen species (ROS) generated by mitochondria, is responsible for MeHg demethylation. Here, we investigated the impact of different mitochondrial respiratory inhibitors, namely rotenone and antimycin A, on the $O_2^{\bullet -}$ mediated degradation of MeHg in human neuroblastoma cells SH-K-SN. We also utilized paraquat (PQ) which generates $O_2^{\bullet -}$ in the mitochondrial matrix. We found that the cleavage of the carbon-metal bond in MeHg was highly dependent on the topology of $O_2^{\bullet -}$ production by mitochondria. Both rotenone and PQ, which increase $O_2^{\bullet -}$ in the mitochondrial matrix at a dose-dependent manner, enhanced the conversion of MeHg to inorganic mercury (iHg). Surprisingly, antimycin A, which prompts emission of $O_2^{\bullet -}$ into the intermembrane space, did not have the same effect even though antimycin A induced a dose dependent increase in $O_2^{\bullet -}$ emission. Rotenone and PQ also enhanced the toxicity of sub-toxic doses (0.1 μM) MeHg which correlated with the accumulation of iHg in mitochondria and depletion of mitochondrial protein thiols. Taken together, our results demonstrate that MeHg degradation is mediated by mitochondrial $O_2^{\bullet -}$, specifically within the matrix of mitochondria when $O_2^{\bullet -}$ is in adequate supply. Our results also show that $O_2^{\bullet -}$ amplifies MeHg toxicity specifically through its conversion to iHg and subsequent interaction with protein cysteine thiols (R-SH). The implications of our findings in mercury neurotoxicity are discussed herein.

1. Introduction

Due to its tendency to bioaccumulate through the food chain MeHg is considered a major public health concern. MeHg has been documented to exert its toxic effects on various organs including the liver, kidney, and cardiac tissue but is for the most part considered a potent neurotoxin (Hansen et al., 1989). This is attributed to its capacity to disrupt brain function, induce neuronal, astrocyte, and microglial cell death, and hinder neurological development (Tofighi et al., 2011; Rodier, 1995). The neurotoxicity of MeHg are due to its physical properties which imbues it with the capacity to easily diffuse through the blood–brain barrier and accumulate in neural tissue (Aschner and Aschner, 1990; Mailloux et al., 2015a). Once inside the cell MeHg can disrupt cellular functions including mitochondrial metabolism, antioxidant defense, and cell signaling pathways culminating with the induction of oxidative stress, oxidative damage, and cell death (Carocci et al., 2014). It is important to point out that the mechanistic details surrounding how MeHg disrupts cellular functions have not been thoroughly investigated. MeHg does harbor soft acid properties which could convey the molecule with the potential to chelate protein cysteine thiols disrupting cellular functions (LoPachin and Barber, 2006). However, there is a considerable latency in observable MeHg neurotoxicity following exposure (Hansen et al., 1989). In addition, following prolonged MeHg exposure the chief Hg species found in brain tissue is inorganic mercury (iHg) (Hansen et al., 1989). This would suggest that the potent toxic effects of MeHg may also be due to its progressive bioconversion to iHg. Due to its charged nature, iHg cannot free diffuse through lipid bilayers and thus readily accumulates in the intracellular environment. In addition, iHg is a strong Lewis acid which instills it with the capacity to bind strongly to cysteine thiols.

Demethylation of MeHg can proceed abiotically by photolytic degradation or via reactions with singlet electron oxygen free radicals like hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) (Fleck et al., 2014; Zhang and Hsu-Kim, 2010). Free radicals like OH• can also be produced in biological systems as a by-product of the singlet electron reduction of di-oxygen (O_2) to H_2O. It has been documented that $OH^{\bullet}$ can biodegrade MeHg in rat liver however; considering OH• is rarely generated in biological systems and when produced it reacts very rapidly with the first molecule it comes in contact with it seems highly unlikely that $OH^{\bullet}$ is responsible for most of MeHg degradation in neural cells (Goldstein and Czapski, 1984; Suda and Hirayama, 1992). By contrast, $O_2^{\bullet -}$, the proximal ROS generated as a consequence of the singlet electron reduction of di-oxygen (O_2), is continuously generated by biological systems since its formation is thermodynamically favorable. This is by virtue of the rapid action of superoxide dismutase (SOD) which metabolizes

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$O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) with extremely rapid kinetics (Mailloux, 2015). The H_2O_2 generated can then be utilized in cell signaling or degraded by a series of redundant antioxidant systems. Thus, in contrast to $OH^{\bullet}$ which is only produced during oxidative stress and occurs at negligible concentrations, other ROS like $O_2^{\bullet-}$ are generated constantly and under certain conditions can display transient increases in production. Recently our group provided direct evidence that $O_2^{\bullet-}$ is able to induce the homolytic cleavage of MeHg generating iHg (Mailloux et al., 2015a). Augmentation of cellular $O_2^{\bullet-}$ production with PQ, which generates the bulk of its $O_2^{\bullet-}$ at the level of Complex I in the mitochondrial matrix, amplifies MeHg degradation which enhances overall Hg toxicity (Mailloux et al., 2015a). We were also able to show that the $O_2^{\bullet-}$ degradation of MeHg could be stopped by adding purified superoxide dismutase (SOD) to reaction mixtures (Mailloux et al., 2015a). Also, we were able to exclude H_2O_2 and $OH^{\bullet}$ as catalysts for MeHg degradation both inside cells and in vitro (Mailloux et al., 2015a). This is in agreement with several previous studies that provided indirect evidence that the bioconversion of MeHg to iHg can induced by rotenone or PQ in primary astrocytes, cell lines, or rat liver slices (Nagano et al., 2010; Shapiro and Chan, 2008). These results are especially relevant under conditions when $O_2^{\bullet-}$ production is elevated either by mitochondrial dysfunction or the presence of other toxins that either 1) self-propagate $O_2^{\bullet-}$ via redox cycling or 2) induce $O_2^{\bullet-}$ production by inhibition of electron transfer reactions in mitochondria.

In the present study we hypothesized that mitochondrial respiratory inhibitors which amplify $O_2^{\bullet-}$ formation also augment the demethylation of MeHg. We utilized rotenone and antimycin A to stimulate high rates of $O_2^{\bullet-}$ from sites I_F and III_Q on the matrix and intermembrane space sides of the mitochondrial inner membrane, respectively (Fig. 5). To our surprise, we found that $O_2^{\bullet-}$ mediated MeHg demethylation is dependent on the topology of mitochondrial $O_2^{\bullet-}$ production. Rotenone induced a dose-dependent increase in the accumulation of iHg with the bulk of being located in mitochondria. The accumulation of iHg was dependent on the concentration of rotenone utilized and the amount of $O_2^{\bullet-}$ produced. Similar results were obtained using paraquat (PQ). Rotenone and PQ also enhanced the toxicity of nontoxic doses of MeHg which is attributed to the accumulation of iHg in mitochondria and subsequent disruption of protein cysteine thiols. Although antimycin A induced a dose-dependent increase $O_2^{\bullet-}$ production it had a limited effect on the bioconversion of MeHg to iHg. Our findings provide mechanistic insight into Hg neurotoxicity showing that MeHg demethylation is carried out in cells by $O_2^{\bullet-}$ and this bioconversion occurs predominantly inside the mitochondria. The implications of our findings for Hg neurotoxicity are discussed herein.

2. Experimental procedures

2.1. Cell culture and dosing

Human neuroblastoma cells were purchased from the American Type Culture and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma) containing 25 mM D-glucose, 4 mM L-glutamine, 1 mM pyruvate, 10% fetal bovine serum (FBS; 10% v/v), and antibiotics–antimycotics (2% v/v). Media was changed every 2 days and cells passaged every 4 days. Cell passage number did not exceed 18. Cells were also routinely cultured and grown in T75 cm^2 matrigel-coated flasks. For experiments cells were then plated at high density in matrigel-coated 96 well plates, 6 welled plates, or 15 cm petri plates to maximize the amount of reduced cellular glutathione (Gutscher et al., 2008). Upon reaching ~90% confluency cells were treated with cell culture media containing MeHg (0–10 μM; stock prepared in PBS) with or without Complex I inhibitor rotenone (0–15 μM; stock prepared in anhydrous ethanol), Complex II inhibitor diethylmalonate (0–25 mM), Complex III inhibitor antimycin A (0–10 μM; stock prepared in anhydrous ethanol), or matrix $O_2^{\bullet-}$ generating molecule paraquat (PQ; 0–500 μM) (Fig. 5). Following a 24 h treatment media was aspirated and cells utilized for assays or mitochondrial isolation. MeHgCl (standard solution in H_2O; 1000 ppm by AA) was obtained from Alfa Aesar (Ward Hill, MA, USA) and dissolved in MilliQ water. MeHg stocks were stored at 4 °C and in the dark to prevent photolytic degradation. Stocks were also routinely monitored for purity.

2.2. Mitochondrial isolation

For each treatment group, three 15 cm petri plates containing cell cultures that were ~90% were combined and utilized for mitochondrial isolation to ensure enough mitochondria were extracted for analysis. All mitochondrial isolation steps were performed on ice or at 4 °C. In addition, all reagents and instruments were cooled on ice or at 4 °C. Cell monolayers were washed once with warmed PBS, trypsinized and centrifuged at 1, 200 rpm for 5 min at room temperature. Following two washes with PBS the cell pellet was resuspended in ice cold mitochondrial isolation buffer (MIB; 220 mM mannitol, 70 mM sucrose, 2 mM dithiothreitol, 1 mM EGTA, 20 mM Hepes, pH 7.0 + 0.2% w/v defatted bovine serum albumin). Note that BSA was added fresh the day of experiments. Cells were disrupted using the Potter–Elvejham method utilizing a glass homogenization tube and Teflon pestle (~30 passes). The homogenate was then transferred to microfuge tubes and centrifuged at 200 ×*g* for 5 min to remove whole cells. The supernatant was collected and centrifuged at 800 ×*g* for 10 min to remove nuclei and then 12,000 ×*g* for 10 min to pellet the mitochondria. The supernatant, which represents the cytosol, was kept for analysis. The mitochondrial pellet was resuspended in 200 μL of MIB devoid of BSA. Protein content of the cytosolic and mitochondrial fractions was determined by Bradford with BSA as a standard.

Purity of mitochondrial and cytosolic fractions was assessed by immunoblot. Mitochondria and cytosol were diluted to 1 mg/mL in Laemmli buffer and then 30 μg of protein was loaded into 10% denaturating isocratic acrylamide gels and electrophoresed. Upon completion, protein was transferred to nitrocellulose membranes by electroblotting and stained with Ponceau S for assessment of transfer efficiency and loading consistency. Membranes were then blocked for 2 h under constant agitation at room temperature with 5% v/v non-fat skim milk solution diluted in tris buffered saline (pH 8.0)/0.1% v/v tween-20 (TBS-T). Membranes were then washed twice with TBS-T and probed overnight with either OXPHOS mitochondrial antibody cocktail (Abcam, 1:1500 in TBS-T supplemented with 5% w/v defatted BSA and 0.02% w/v NaN_3) or GADPH antiserum (Sigma, 1:5000). The OXPHOS antibody cocktail consists of NDUFB8, succinate dehydrogenase B, UQCRC2, MTCO1, and ATP5A antiserum. Blots were then washed twice and probed with anti-rabbit HRP conjugate (Santa Cruz, 1/3000 in non-fat skim milk). Bands were visualized using Supersignal West Pico Chemiluminescent substrate and documented.

2.3. Mercury extraction and analysis

MeHg and iHg were extracted simultaneously from cells or cytosol and mitochondria using nitric acid as described in Barst et al. (2013). For cells, following exposure to MeHg and rotenone, PQ, or antimycin A cells were washed three times with warmed PBS to remove any residual Hg (MeHg and iHg) and then treated immediately with 2 mL of 4.5 N nitric acid solution prepared in MilliQ water. Cells were then scrapped from the surface the plates and then incubated overnight at 60 °C. Solutions were transferred into 20 mL screw top vials containing 5 mL methylene chloride and shaken vigorously overnight at room temperature. The upper nitric acid phase which contains iHg was frozen at −80 °C. The lower methylene chloride phase was placed in 7 mL glass screw top vials containing 1 mL 0.01 M L-cysteine prepared in a 2% v/v solution of HNO_3 to reverse extract MeHg. The upper L-cysteine phase containing MeHg was then collected and stored at −80 °C. MeHg and iHg levels were monitored in a MA-3000 Mercury Analyzer (Nippon Instrument, Japan). Levels were determined using standard curves developed