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Expression of human oxoguanine glycosylase 1 or formamidopyrimidine glycosylase in human embryonic kidney 293 cells exacerbates methylmercury toxicity *in vitro*

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ABSTRACT

Exposure to methylmercury (**MeHg**) acutely at high levels, or via chronic low-level dietary exposure from daily fish consumption, can lead to adverse neurological effects in both the adult and developing conceptus. To determine the impact of variable DNA repair capacity, and the role of reactive oxygen species (**ROS**) and oxidatively damaged DNA in the mechanism of toxicity, transgenic human embryonic kidney (**HEK**) 293 cells that stably express either human oxoguanine glycosylase 1 (**hOgg1**) or its bacterial homolog, formamidopyrimidine glycosylase (**Fpg**), which primarily repair the oxidative lesion 8-oxo-2′-deoxyguanosine (**8-oxodG**), were used to assess the *in vitro* effects of MeHg. Western blotting confirmed the expression of hOgg1 or Fpg in both the nuclear and mitochondrial compartments of their respective cell lines. Following acute (1–2 h) incubations with 0–10 μM MeHg, concentration-dependent decreases in clonogenic survival and cell growth accompanied concentration-dependent increases in lactate dehydrogenase (**LDH**) release, ROS formation, 8-oxodG levels and apurinic/apyrimidinic (**AP**) sites, consistent with the onset of cytotoxicity. Paradoxically, hOgg1- and Fpg-expressing HEK 293 cells were more sensitive than wild-type cells stably transfected with the empty vector control to MeHg across all cellular and biochemical parameters, exhibiting reduced clonogenic survival and cell growth, and increased LDH release and DNA damage. Accordingly, upregulation of specific components of the base excision repair (**BER**) pathway may prove deleterious potentially due to the absence of compensatory enhancement of downstream processes to repair toxic intermediary abasic sites. Thus, interindividual variability in DNA repair activity may constitute an important risk factor for environmentally-initiated, oxidatively damaged DNA and its pathological consequences.

Introduction

Methylmercury (MeHg) is a potent neurotoxin that persists in the environment. It is most notorious for the more than 900 human fatalities that resulted in Minamata Bay, Japan, from the consumption of MeHg-contaminated seafood that contained up to 40 ppm (≈184 μM MeHg (National Institute for Minamata Disease, Harada, 1995). Today however, society's attempt to foster a healthier lifestyle by increasing their dietary intake of fish and other seafood is posing a less catastrophic but more prevalent health threat with some widely consumed species of fish, including swordfish, shark, pike and bass, harboring high concentrations of MeHg, in some greater than 1 ppm (≈4.6 μM MeHg) (Health Canada). Our studies herein have been carried out at working concentrations of MeHg well below the range associated with fatalities, and within the more commonly relevant concentration range (≈0.1–9 μM MeHg) found in various fish and seafood species.

The developing nervous system appears to be particularly susceptible to MeHg, with *in utero* MeHg exposure associated with a range of structural and functional postnatal neurodevelopmental deficits (Castoldi et al., 2003; Choi, 1989; Davis et al., 1994). A number of mechanisms have been postulated to explain the potent neurotoxic action of MeHg. Among them, the role of oxidative stress and its resulting pathological consequences has garnered much of the attention, yet the precise molecular mechanisms remain to be elucidated. Various groups have demonstrated the reactive oxygen species (ROS)-initiating ability of MeHg, its capacity to damage DNA and attenuation of toxicity with antioxidant

Abbreviations: 8-oxodG, 8-oxo-2′-deoxyguanosine; AP, apurinic/apyrimidinic; APE1, AP endonuclease 1; ARP, aldehyde reactive probe; BCNU, 1,3-N,N′-bis(2-chloroethyl)-N-nitrosourea; BER, base excision repair; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; DCF, dichlorodihydrofluorescein; dG, deoxyguanosine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyromidine; Fpg, formamidopyrimidine glycosylase; HEK, human embryonic kidney; hOgg1, human oxoguanine glycosylase 1; HPLC-UV, high-performance liquid chromatography with detection by ultraviolet absorbance; LDH, lactate dehydrogenase; LIG3α, DNA ligase 3α; MeHg, methylmercury; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; MYH, MutY homolog; Ogg1, oxoguanine glycosylase 1; *Ogg1*$^{-/-}$, Ogg1-*null*; POLβ, DNA polymerase β; ROS, reactive oxygen species; TBST, 0.1% Tween-20 in 1X Tris-buffered saline; XRCC1, X-ray cross-complementing 1.

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pretreatment (Ali et al., 1992; Belletti et al., 2002; Chen et al., 2005; Garg and Chang, 2006; Jie et al., 2007; Ondovcik et al., 2012; Rush et al., 2012; Shanker et al., 2005). Thus, considerable evidence supports a potential role for ROS-mediated oxidatively damaged DNA in the mechanism of MeHg toxicity.

The base excision repair (BER) pathway is one highly conserved form of DNA repair responsible for maintaining both genomic stability and integrity. It does so by removing aberrant bases from the genome, and is the pathway primarily responsible for removal of oxidatively damaged bases such as 8-oxo-2′-deoxyguanosine (8-oxodG), which if not repaired can lead to potentially carcinogenic G:C to T:A transversion mutations, as well as interfere with transcriptional machinery altering gene expression (Khobta et al., 2010; Kitsera et al., 2011; Klungland and Bjelland, 2007; Klungland et al., 1999; Pastoriza-Gallego et al., 2007), the latter of which may be particularly important for normal brain development (Wells et al., 2010). BER-mediated repair of 8-oxodG is initiated by the bifunctional DNA glycosylase/apurinic/apyrimidinic (AP) lyase oxoguanine glycosylase 1 (Ogg1) in mammalian cells, and its functional homolog in bacteria, formamidopyrimidine glycosylase (Fpg) (Klungland et al., 1999). Purified Fpg is catalytically more efficient than Ogg1 *in vitro*, excising 8-oxodG and 2,6-diamino-4-hydroxy-5-formamidopyromidine (FapyG) from oligonucleotide constructs 80-fold faster as evidenced by its higher k_{cat}/K_m values; a phenomenon attributed to higher substrate affinity and quicker intermediate hydrolysis, whereby Fpg releases product more readily from its catalytic incision site than does Ogg1 (Asagoshi et al., 2000; Frosina, 2006). These glycosylases act to recognize and excise the damaged base creating an AP site, which can be cytotoxic without the action of the downstream short-patch BER machinery consisting of AP endonuclease 1 (APE1), DNA polymerase β (POLβ), DNA ligase 3α (LIG3α) and X-ray cross-complementing 1 (XRCC1), all of which work in concert to complete the repair process (David et al., 2007; Wilson and Bohr, 2007).

The importance of DNA repair in disease has been underscored by observations in both animals and humans where deficiencies have proved deleterious. Ogg1-*null* (*Ogg1*$^{-/-}$) mice accumulate hepatic 8-oxodG corresponding to a 2-3-fold increased spontaneous mutation frequency (Klungland et al., 1999; Minowa et al., 2000); display increased postnatal neurodevelopmental deficits following *in utero* exposure to the ROS-initiating drug methamphetamine (Wong et al., 2008); and, with the combined absence of MutY homolog (MYH) responsible for excising adenine misincorporated opposite 8-oxodG during replication, have an increased incidence of lung and small intestine cancer (Russo et al., 2004). In humans, the serine to cysteine polymorphism at amino acid residue 326 of the Ogg1 protein compromises repair activity and has been associated with an increased cancer incidence (Goode et al., 2002; Hung et al., 2005). However, whether enhancement of BER capacity through overexpression of Ogg1 or Fpg is protective remains unclear with conflicting reports in the literature. Overexpression of human Ogg1 (hOgg1) in the mitochondria of human hepatoma cells exacerbated cisplatin-mediated cytotoxicity (Zhang et al., 2007), while the overexpression of *N*-methylpurine DNA glycosylase in human breast cancer cells similarly sensitized them to the alkylating agent methyl methanesulfonate (Rinne et al., 2004). In contrast, expression of hOgg1 or Fpg in human lung epithelial cells protected against 1,3-N, N′-bis(2-chloroethyl)-N-nitrosourea (BCNU)-mediated DNA damage and cytotoxicity (He et al., 2002), as did their expression in human embryonic kidney (HEK) 293 cells against 8-oxodG accumulation and cytotoxicity initiated by menadione, cisplatin and oxaliplatin (Preston et al., 2009).

The ROS-initiating and DNA-damaging abilities of MeHg likely are key players in its mechanism of toxicity. However, no studies had examined the effects of variable BER capacity on MeHg toxicity prior to our first report of an increased sensitivity of *Ogg1*$^{-/-}$ cells to MeHg (Ondovcik et al., 2012). Thus, we sought to further evaluate the role of altered BER capacity and the contribution of ROS and oxidatively damaged DNA in the mechanism of MeHg toxicity in a reciprocal model of enhanced BER, employing HEK 293 cells engineered in our lab to stably express hOgg1 or Fpg. Herein we provide the first report of an altered sensitivity of hOgg1- and Fpg-expressing cells to MeHg-initiated cytotoxicity, with a paradoxical increase in sensitivity likely resulting from their increased incision activity and subsequent accumulation of toxic repair intermediates. Our results support a role for ROS-mediated oxidatively damaged DNA in the mechanism of MeHg toxicity, and implicate interindividual variability in DNA repair activity as a potential determinant of risk.

Materials and methods

Cell culture. Wild-type human embryonic kidney (HEK) 293 cells stably transfected with the empty vector control, and HEK cells expressing human oxoguanine glycosylase 1 (hOgg1) or formamidopyrimidine glycosylase (Fpg) were obtained and derived in our laboratory as previously described (Preston et al., 2009). Cells were grown at 37°C in 5% CO_2 (The Linde Group, Etobicoke, Ontario) in a humidified incubator, and maintained in Minimum Essential Medium Alpha containing 2 mM L-glutamine (Life Technologies Inc., Burlington, Ontario), and supplemented with 1200 μg/ml G418 (Geneticin) disulfate salt solution, 1% penicillin/streptomycin solution (both Sigma-Aldrich Canada Ltd., Oakville, Ontario) and 10% heat-inactivated fetal bovine serum (HyClone, Thermo Fisher Scientific, Ottawa, Ontario).

Anti-FLAG-mediated western blotting for hOgg1-FLAG and Fpg-FLAG protein expression. Nuclear and mitochondrial protein extracts were prepared from wild-type empty vector control, hOgg1- and Fpg-expressing HEK 293 cells as described previously (Dobson et al., 2000). Ten micrograms of each nuclear and mitochondrial protein lysate were loaded onto a 10% polyacrylamide gel, run for 2 h at 50 V, and transferred onto a nitrocellulose membrane at 60 V for 1.5 h. The membrane was blocked in 0.1% Tween-20 in 1X Tris-buffered saline (TBST) containing 3% skim milk and incubated overnight at 4 °C with an anti-FLAG M2 mouse monoclonal antibody (1:5000 in TBST containing 1% skim milk) (Sigma-Aldrich Canada Ltd.), followed by incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:2000 in TBST) (Santa Cruz Biotechnology Inc., Santa Cruz, California). Detection was accomplished using enhanced chemiluminescent detection reagents (Amersham, GE Healthcare, Baie d'Urfe, Quebec) and exposure onto Bioflex MSI film (Clonex, Markham, Ontario).

Clonogenic assay. Five hundred cells were seeded into six-well plates and treated the following day for 1 h with 0–10 μM of methylmercury (II) chloride (MeHg, Sigma-Aldrich Canada Ltd.). Surviving cells were allowed to grow into visible colonies for 8–10 days post-treatment, and were then fixed and stained with 0.5% methylene blue in 100% methanol for 15 min. Clonogenic survival was determined from the mean number of colonies plus the standard deviation of three independent determinations, and is presented as a percentage of untreated cells.

Assessment of cell growth via Hoechst bisbenzimide 33258. One thousand cells were seeded into 96-well plates, allowed to adhere overnight, and left untreated or treated for 1 h with 10 μM MeHg. In an additional 96-well plate per line, known cell numbers (0–50,000) were seeded to generate a standard curve from which cell numbers would be determined via linear regression analysis at the various days post-treatment. Cells were washed with Milli-Q water and frozen at −80 °C at 0, 2, 4, 6 and 8 days post-treatment, after which they were stained with 20 μg/ml Hoechst bisbenzimide 33258 (Sigma-Aldrich Canada Ltd.). Fluorescence data were measured and analyzed using a SpectraMax Gemini XS microplate spectrofluorometer (excitation, 350 nm; emission, 460 nm) and SoftMax Pro 5.4 software respectively (both MDS Analytical Technologies, Sunnyvale, California).