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Toxicological effects of thiomersal and ethylmercury: Inhibition of the thioredoxin system and NADP$^+$-dependent dehydrogenases of the pentose phosphate pathway

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ABSTRACT

Mercury (Hg) is a strong toxicant affecting mainly the central nervous, renal, cardiovascular and immune systems. Thiomersal (TM) is still in use in medical practice as a topical antiseptic and as a preservative in multiple dose vaccines, routinely given to young children in some developing countries, while other forms of mercury such as methylmercury represent an environmental and food hazard. The aim of the present study was to determine the effects of thiomersal (TM) and its breakdown product ethylmercury (EtHg) on the thioredoxin system and NADP$^+$-dependent dehydrogenases of the pentose phosphate pathway. Results show that TM and EtHg inhibited the thioredoxin system enzymes in purified suspensions, being EtHg comparable to methylmercury (MeHg). Also, treatment of neuroblastoma and liver cells with TM or EtHg decreased cell viability (GI_{50}: 1.5 to 20 μM) and caused a significant ($p < 0.05$) decrease in the overall activities of thioredoxin (Trx) and thioredoxin reductase (TrxR) in a concentration- and time-dependent manner in cell lysates. Compared to control, the activities of Trx and TrxR in neuroblastoma cells after EtHg incubation were reduced up to 60% and 80% respectively, whereas in hepatoma cells the reduction was almost 100%. In addition, the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also significantly inhibited by all mercurials, with inhibition intensity of $Hg^{2+} > MeHg \approx EtHg > TM$ ($p < 0.05$). Cell incubation with sodium selenite alleviated the inhibitory effects on TrxR and glucose-6-phosphate dehydrogenase. Thus, the molecular mechanism of toxicity of TM and especially of its metabolite EtHg encompasses the blockage of the electrons from NADPH *via* the thioredoxin system.

Introduction

Mercurial compounds have shown a wide range of toxicological effects on human beings, involving especially the central nervous system, causing damage to the brain, but also to the kidneys, the cardiovascular and immune systems (Clarkson et al., 2003; Dórea et al., 2013). Exposure to mercurial compounds such as methylmercury (MeHg) and mercuric mercury (Hg^{2+}) at levels above the toxicity threshold occurs either by regular fish consumption or occupational contact, respectively, and represents a major concern in toxicology (Clarkson et al., 2003; Carvalho et al., 2008a; Nunes et al., 2014). Not less important is mercury exposure in dental practice for both dentists and patients due to the use of dental amalgam fillings that release mercury vapour (Clarkson et al., 2003). Even though the use of mercury compounds such as thiomersal (TM) in medicines and antiseptics is decreasing it is still used as a preservative in some formulas, namely in vaccines (Sykes et al., 2014).

Although mercurial compounds are not new toxicants, there is a significant lack of knowledge about their molecular mechanisms of toxicity, especially about TM and its breakdown product ethylmercury (EtHg). TM, a mercury derivative composed of EtHg and thiosalicylic acid has been widely used as a preservative in vaccines, dermatological (topic) and ocular preparations. Indeed, vaccines with TM are the main route of mercury exposure in clinics (Bigham and Copes, 2005) and while children in most of the developed countries receive normally TM-free vaccines, children in developing countries may receive several doses of different

Abbreviations: 6PGDH, 6-phosphogluconate dehydrogenase; EtHg, ethylmercury; G6PDH, glucose-6-phosphate dehydrogenase; Hg^{2+}, mercuric mercury; MeHg, methylmercury; Se^{2-}, selenide; SeO_3^{2-}/Se (IV), selenite; Se, selenium; TCV, thiomersal-containing vaccines; TM, thiomersal; Trx, thioredoxin; TrxR, thioredoxin reductase.

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TM-containing vaccines (TCV) (Sykes et al., 2014; Dórea and Marques, 2008). TM is degraded in the organism releasing EtHg that is more toxic than the parent compound and thus, TCV constitute the main route of exposure of human populations to this organomercurial (Clarkson et al., 2003). Experimental studies that addressed early life exposure to TCV indicate that in exposed foetuses, neonates and infants, mercury remains in their blood at sufficient concentration and for enough time to reach the brain and to exert a neurologic impact and affect neurodevelopment (Dórea and Marques, 2008; Dórea et al., 2013).

Recent epidemiological studies evaluated exposure to different mercurials and found that even low levels of mercury cause subtle adverse effects on brain functions (Barboni et al., 2009; Debes et al., 2006) and may interfere with neurobehavioral development in children and affect also the immune system and cause progression of atherosclerosis and cardiovascular diseases in adults (Kjellström et al., 1989; Shamlaye et al., 1995; Vas and Monestier, 2008; Virtanen et al., 2005). However, the mechanism of toxicity has not been well understood and studies with EtHg and TM are scarce *in vivo* and *in vitro* (Geier et al., 2009; Ida-Eto et al., 2013; Migdal et al., 2010a,b; Mutkus et al., 2005; Pieper et al., 2014; Sharpe et al., 2012; Westphal et al., 2003). In this context, the better understanding of the molecular mechanism of action of TM and EtHg besides being a priority in toxicology research would help to clarify the epidemiological controversy around TCV and mercury intoxication.

It has been demonstrated that inhibition of the thioredoxin system, is one of the main mechanisms of Hg^{2+} and MeHg toxicity *in vitro* and *in vivo* (Branco et al., 2011; Carvalho et al., 2008b, 2011) but there are no studies concerning the interaction between TM or EtHg with this enzymatic cycle, especially in the central nervous system. This cycle is composed by two enzymes: thioredoxin (Trx) and thioredoxin reductase (TrxR) that act together with NADPH as a co-factor to reduce oxidized protein substrates. This system is responsible for maintaining the general reduced state in cells being a major representative in antioxidant defence (Lu and Holmgren, 2014; Kohárýová and Kolárová, 2008). Additionally, the thioredoxin system plays a key role in many physiological processes such as DNA synthesis, apoptosis regulation and redox signalling, being involved in the development of many pathologies (Joshi et al., 2014; Lu and Holmgren, 2014). Since the thioredoxin system is upstream of a network of pathways related to cell survival and proliferation, its inhibition as an early event would trigger a series of downstream cellular signalling that can lead to cell death (Lillig and Holmgren, 2007).

NADPH is mostly generated by the pentose phosphate pathway (PPP) being one of the main intracellular reducing agents and an essential co-factor required for the normal function of antioxidant cycles such as the glutathione and thioredoxin systems (Horecker, 2002). The PPP is a main route for biosynthesis of nucleic acids and aromatic amino acids as well as glucose catabolism. In the oxidative phase of the PPP the thiol-containing enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) generate NADPH + H^+. Targeting of these enzymes by mercury compounds would decrease NADPH and further affect the thioredoxin cycle performance.

Sodium selenite (Na_2SeO_3), has been used as a protective agent for mercury toxicity, since Se(IV) can be reduced by the mammalian selenoenzyme TrxR to selenide (Se^{2-}), which has high affinity for Hg^{2+} and is able to remove it from the active site of TrxR, forming mercury selenide (HgSe) (Carvalho et al., 2011). As recently demonstrated (Branco et al., 2014), the protective effect of selenite on Hg^{2+} toxicity is also due to the up-regulation of TrxR1 expression *via* the Nrf-2 pathway.

Therefore, this study aimed at investigating if TM and EtHg inhibit the thioredoxin system and the pentose phosphate pathway using both purified enzyme and human cell cultures. Another goal was to verify if selenite supplementation could antagonize the detrimental effects of TM and EtHg.

Methods

Materials and enzymes. Human recombinant insulin, GSH, DTNB, mercuric chloride ($HgCl_2$), methylmercury chloride (MeHgCl), ethylmercury chloride (EtHgCl), and thiomersal (ethyl(2-mercaptobenzoate-(2-)-O,S) mercurate (1-) sodium) (TM) were purchased from Sigma (St. Louis, MO, USA). TM and $HgCl_2$ were freshly prepared in Milli-Q water while MeHg and EtHg were prepared in DMSO, all of them as 100 mM stock solutions.

The preparation of recombinant rat TrxR and wild-type human Trx (IMCO Corp., Sweden) was carried out as previously described by Arnér et al. (1999). The enzymatic activity of wild-type TrxR was determined by the DTNB assay before its use, and concentrations mentioned are reported for active enzyme. Human Trx was DTT-treated to obtain the fully reduced form which was followed by desalting on NAP-5 columns (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with 50 mM Tris·Cl:1 mM EDTA (TE) buffer (pH 7.5), to remove excess DTT.

TrxR activity determination by insulin reduction assay using purified enzymes. Activity of TrxR was determined with a modification of a previous protocol (Carvalho et al., 2011). In 96-well plates, 10 nM recombinant rat TrxR was preincubated with $HgCl_2$, MeHgCl, TM and EtHgCl at different concentrations (0–1 μM) and NADPH (1.65 μM) in 50 mM Tris–HCl pH 7.5 for 5 min at room temperature. Then, 2 μM hTrx was added before a mix solution containing insulin and NADPH (final concentration 160 and 200 μM, respectively) in Tris buffer. Absorbance at 340 nm was followed in a microplate reader (Zenyth 3100, Anthos Labtec Instruments) and activity was calculated as the linear change in absorbance over the initial 15 min.

Cell culture. HepG2 and SH-SY5Y cells were cultured in DMEM/F-12 (1:1) medium, supplemented with 10% FBS and penicillin/streptomycin (5%) (from Gibco) and 2.5% HEPES was added to HepG2 medium. Cell assays and passages were performed at approximately 80% confluence.

Cell viability assay. 4×10^3 HepG2 or SH-SY5Y cells were plated in 96-microwell plates and allowed to grow in appropriate medium, at 37 °C and 5% CO_2 for 24 h. Then, the medium was changed to the growth medium containing TM or EtHg at different concentrations (TM or EtHg: 0.5–100 μM for HepG2 cells and 0.1–50 μM for SH-SY5Y cells) and time incubations (0, 24, 48 and 72 h). Cell viability was measured by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mosmann, 1983).

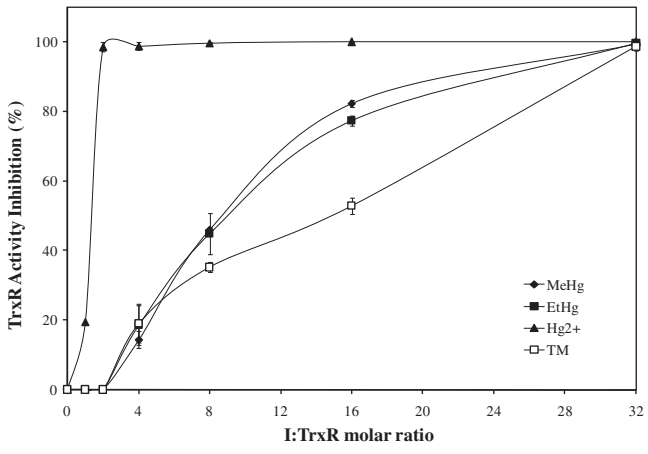

Fig. 1. Effect of mercurial compounds (25, 50, 100, 200 and 400 nM) on purified thioredoxin reductase (10 nM). Results are expressed as the mean ± SEM of three different experiments. MeHg: methylmercury; EtHg: ethylmercury; Hg^{2+}: mercuric mercury TM: thiomersal.