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In vivo and in vitro inhibition of mice thioredoxin reductase by methylmercury

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Abstract The thioredoxin (Trx) system, involving redox active Trxs and thioredoxin reductases (TrxRs), sustain a number of important Trx-dependent pathways. These redox active proteins support several processes crucial for cell function, cell proliferation, antioxidant defense, and redox-regulated signaling cascades. Methylmercury (MeHg) is an important environmental toxicant that has a high affinity for thiol groups and can cause oxidative stress. The Trx system is the major system responsible for maintaining the redox state of cells and this function involves thiol reduction mediated by selenol groups in TrxRs. MeHg has a great affinity to thiols and selenols, thus the potential toxic effects of MeHg on TrxR inhibition were determined in the current study. A single administration of MeHg (1, 5, and 10 mg/Kg) caused a marked inhibition of kidney TrxR activity, while significant inhibition was observed in the liver after exposure to 5 and 10 mg/Kg of MeHg. TrxR activity was determined 24 h after MeHg. In the brain, MeHg did not inhibit TrxR activity. In vitro exposure to MeHg indicated that MeHg inhibits cerebral (IC_{50}, 0.158 μM), hepatic (IC_{50}, 0.071 μM), and renal TrxR activity (IC_{50}, 0.078 μM). The results presented herein demonstrated for the first time that renal and hepatic TrxRs can serve as an in vivo target for MeHg. This study suggests that MeHg can bind to selenocysteine residues present in the catalytic site of TrxR, in turn causing enzyme inhibition that can compromise the redox state of cells.

Keywords Thioredoxin reductase · Methylmercury toxicity · Thiol · Enzyme inhibition · Kidney damage

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Introduction

The thioredoxin (Trx) system, composed of thioredoxin reductase (TrxR), Trx, and NADPH, plays an important role in regulating redox metabolism and other cellular processes, such as DNA synthesis, cell proliferation, and apoptosis. This system is widely distributed in different mammalian organs and tissues (Rozell et al. 1985) and is critical for the cellular stress response, protein repair, and protection against oxidative damage (Arner and Holmgren 2000; Lillig and Holmgren 2007).

Human Trx has a conserved dithiol active site, Cys32-Gly-Pro-Cys35, and contains three structural cysteine residues (Cys62, Cys69, and Cys73). These additional residues make Trx susceptible to oxidation via generation of a second disulfide (Cys62–Cys69), which leads to loss of catalytic activity (Holmgren

1985; Lillig and Holmgren 2007; Watson et al. 2003). Trx is the major disulfide reductase responsible for maintaining cytosolic proteins in their reduced state (Fang and Holmgren, 2006).

Mammalian TrxR is a selenoenzyme containing a unique, catalytically-active selenolthiol/selenenylsulfide in the conserved C-terminal sequence (-Gly-Cys-Sec-Gly; Zhong et al. 2000; Sandalova et al. 2001). TrxR has a broad range of functions; specifically, TrxR exhibits broad substrate specificity, reducing many low molecular compounds, including hydrogen peroxide, lipid hydroperoxides, ascorbate, lipoic acid, ubiquinone and Trx (Li et al. 2008). Of particular importance, it has been reported that inhibition of mammalian TrxR can cause deleterious cellular effects, leading to cytotoxicity and cell death (Carvalho et al. 2008; Du et al. 2009).

Mercury contamination is a critical public health problem. Mercury is considered one of the most toxic metals in the environment. Inorganic mercury exists in different oxidation states, and inorganic mercury can be converted to organic mercury by aquatic microorganisms (Clarkson and Magos 2006). Differences in the reactivity and transport of the different forms of mercury are responsible for variations in tissue and organ distribution, patterns of biological effects, and toxic potencies (Clarkson and Magos 2006; de Freitas et al. 2009). The kidneys are the primary target organ for inorganic Hg(II); whereas organic mercuric compounds, such as methylmercury (MeHg), are strong neurotoxicants, primarily damaging the central nervous system (Clarkson et al. 2003; Fonfria et al. 2005; Roos et al. 2009; Aschner et al. 2007; Wagner et al. 2010).

Mercury toxicity has been related to the formation of stable complexes with sulfhydryl-containing molecules, such as the cysteine residues of proteins and non-protein molecules (Ballatori 2002; Rooney 2007). Methylmercury can disrupt cellular redox balance in human monocytes, thus triggering a decrease in Trx1 levels (Wataha et al. 2008). Recently, it has been demonstrated that MeHg can directly inhibit TrxR and Trx activity in HeLa and HEK 293 cells (Carvalho et al. 2008). Horai et al. (2008) have suggested that a change in TrxR levels may be an important mechanism by which Hg can cause hepatic toxicity.

Despite the apparent involvement of disruption of the redox system in MeHg toxicity and the capacity of mercury compounds to interact with thiols and selenols, reports on MeHg interactions with the Trx system are limited. Of particular importance, there are no reports on in vivo MeHg poisoning exploiting TrxR as the molecular end point of MeHg toxicity. Therefore, the objective of this study was to investigate the effects of in vivo exposure to MeHg in cerebral, renal, and hepatic TrxR activity in adult mice. For comparative purposes, we also determined the in vitro effect of MeHg on TrxR from liver, brain, and kidney.

Materials and methods

Chemicals

Methylmercury (II) chloride was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of the highest available commercial grade.

Animals

Male mice (2 months) were maintained under standard conditions (12-h light/dark, $22 \pm 2°C$) with food and water ad libitum. The Animal Care Committee approved all animal handling and experimental conditions.

In vivo treatment

Sixteen mice were equally divided into four experimental groups: control; MeHg 1 mg/kg; MeHg 5 mg/kg; and MeHg 10 mg/kg. The mice were treated by oral gavage. Control mice received a single oral dose of water. After 24 h of administration, the animals were sacrificed. The livers, brains, and kidneys were quickly removed from the mice, placed on ice, and homogenized.

Tissue preparation

Thioredoxin reductase was partially purified by a modification of the method described by Holmgren and Bjornstedt (1995). Tissues were homogenized in buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4; and 1.4 mM KH_2PO_4 [pH = 7.3]). Livers (0.5 g) were homogenized in 10 volumes of buffered saline, brains (0.5 g) were homogeneized in

3 volumes of buffered saline, and kidneys (0.5 g) were homogeneized in 5 volumes of buffered saline. Homogenates were centrifuged at 13,000 *g* for 30 min. The protein in the supernatant was measured and adjusted to 10 mg/ml. The supernatant was dialyzed against buffered saline for 16 h to remove endogenous glutathione and Trx. The dialyzate was heated at 55°C for 10 min, cooled, and centrifuged at 13,000 *g* for 30 min to remove denatured protein.

TrxR activity

Thioredoxin reductase activity was measured by the method described by Holmgren and Bjornstedt (1995). The reaction mixture consisted of the following (in mM): 0.24 NADPH, 10 EDTA, 100 potassium phosphate buffer (pH 7.0), 2 mg/ml DTNB, and 0.2 mg/ml of BSA. The partially purified TrxR was added (to final concentration of 6–8 μg of protein) containing the reaction mixture and the absorbance were followed at 412 nm for a maximum of 4 min.

In vitro assays

Mice were decapitated, and the liver, brain, and kidneys were rapidly removed and homogenized in buffered saline according to the tissue preparation procedure (vide supra). The TrxR activity was determined as described above, with the exception that MeHg was added to the reaction medium (final MeHg concentrations were 0.025, 0.05, 0.075, 0.1, 0.5, or 1 μM).

Protein estimation

The protein concentration was measured by the Lowry method (1951) using bovine serum albumin (BSA) as a standard.

Statistical analysis

The results are expressed as the mean ± standard error, and were analyzed by one-way analysis of variance (ANOVA). The Duncan's multiple range test was applied post hoc to determine the significance of the difference between the various groups. Differences were considered statistically significant at a $P < 0.05$.

Results

In vitro inhibitory effects of MeHg

To evaluate the inhibitory potency of MeHg on TrxR activity, the partially purified enzyme from liver, kidney, and brain was incubated in the presence and absence of MeHg. In liver (Fig. 1), MeHg (0.05, 0.1, 0.5, and 1 μM) caused a significant inhibition of TrxR in a concentration-dependent manner. The IC_{50} values (0.071 μM ± 0.005) for TrxR inhibition by MeHg in liver tissue was the lowest of the three tissues tested (Table 1).

In brain (Fig. 2), MeHg (0.05, 0.1, 0.5, and 1 μM) caused a statistically significant inhibition in TrxR, but with significantly lower potency than determined for liver and kidney. The IC_{50} value for inhibition of brain TrxR was 0.158 μM ± 0.029 (Table 1).

In kidney (Fig. 3), MeHg significantly inhibited TrxR (as low as 0.025 μM) in a concentration-dependent manner. The IC_{50} value was 0.078 μM ± 0.011 (Table 1).

In vivo inhibitory effects of MeHg

To analyze the behavior of MeHg as a potential in vivo inhibitor of TrxR, we exposed mice to three

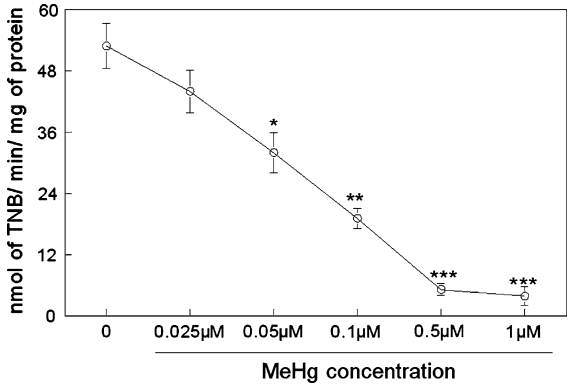

Fig. 1 In vitro inhibition of liver TrxR by different MeHg concentration. MeHg was incubated in reaction medium in different concentrations:0.025, 0.05, 0.1, 0.5 and 1 μM and the reaction were started with the addition of purified enzyme. Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

Table 1 IC_{50} values of in vitro inhibition of TrxR activity by MeHg in different tissues

Tissue	IC_{50} values (μM)
Liver	0.071 ± 0.005
Brain	0.158 ± 0.029*
Kidney	0.078 ± 0.011

Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. * represent statistical differences between the tissues. (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

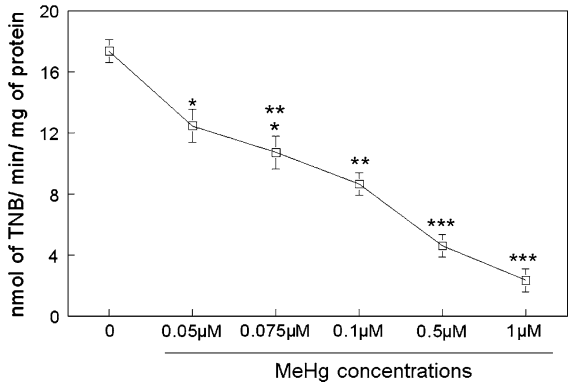

Fig. 2 In vitro inhibition of brain TrxR by different MeHg concentration. MeHg was incubated in reaction medium in different concentrations: 0.05, 0.075, 0.1, 0.5, and 1 μM and the reaction were started with the addition of purified enzyme. Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

different doses of MeHg (1, 5, and 10 mg/kg), and the activity of partially purified TrxR from liver, kidney, and brain was evaluated 24 h after MeHg administration. MeHg caused a significant inhibitory effect on liver TrxR activity in doses of 5 and 10 mg/kg (Fig. 4). Methylmercury, at a dose of 1 mg/kg, decreased TrxR activity, but not statistically different from the control group values (Fig. 4). In contrast to liver and kidney, MeHg treatment did not cause alteration in cerebral TrxR (Fig. 5). In kidney, MeHg caused a marked inhibition of TrxR activity at all doses. Furthermore, MeHg at a dose of 10 mg/kg caused a pronounced inhibitory effect of approximately 80% (Fig. 6).

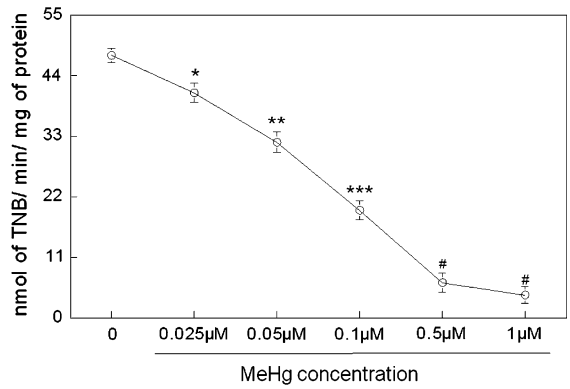

Fig. 3 In vitro inhibition of kidney TrxR by different MeHg concentration. MeHg was incubated into reaction medium in different concentrations:0.0.025, 0.05, 0.1, 0.5, and 1 μM and the reaction were started with the addition of purified enzyme. Data are expressed as mean ± S.E.M. and are calculated for fourth independent assays. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

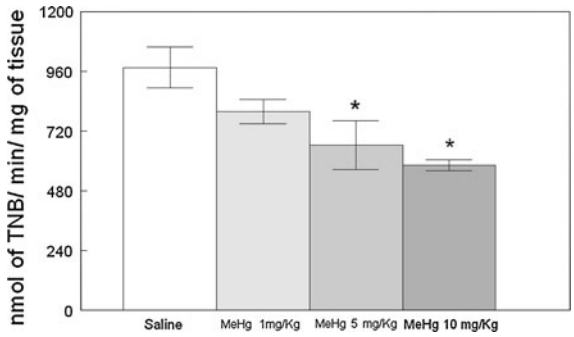

Fig. 4 Inhibition of liver thioredoxin reductase (TrxR) activity by MeHg exposure. Animals were exposed to tree different MeHg concentration: 1, 5, and 10 mg/Kg, during 24 h. TrxR activity was assessed by DTNB reduction method. Data are expressed as mean ± S.E.M. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

Discussion

This study showed that MeHg can effectively inhibit TrxR after in vitro and in vivo exposure. However, the inhibitory potency varied depending on the type of exposure and on the tissue. Indeed, in vivo treatment with MeHg (1–10 mg/kg) inhibited renal TrxR and in vitro experiments confirmed that MeHg

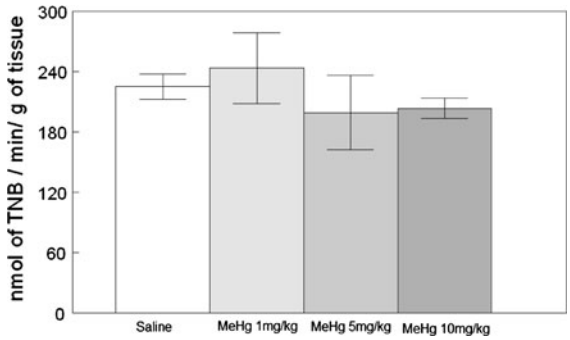

Fig. 5 Inhibition of brain thioredoxin reductase (TrxR) activity by MeHg exposure. Animals were exposed to tree different MeHg concentration: 1, 5, and 10 mg/Kg, during 24 h. TrxR activity was assessed by DTNB reduction method. Data are expressed as mean ± S.E.M. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

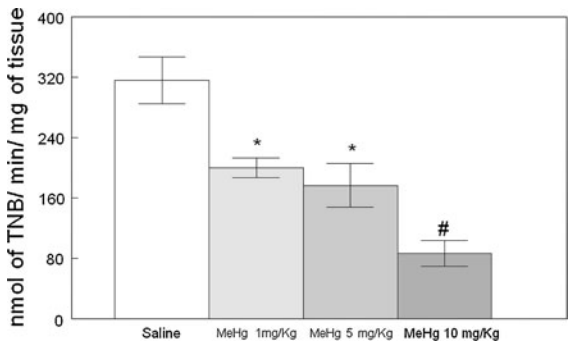

Fig. 6 Inhibition of kidney thioredoxin reductase (TrxR) activity by MeHg exposure. Animals were exposed to tree different MeHg concentration: 1, 5, and 10 mg/Kg, during 24 h. TrxR activity was assessed by DTNB reduction method. Data are expressed as mean ± S.E.M. Distinction between the symbols represent a statistical differences between the groups (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

inhibits this important redox regulatory enzyme. Taking into account that mercuric ions have a greater affinity to bind to reduced sulfur atoms, especially those on endogenous thiol-containing molecules (glutathione, metallothionein, homocysteine, and *N*-acetylcysteine; Hultberg et al. 2001), the biological effects of MeHg can be related to interactions with sulfhydryl-containing residues in the active site of TrxR. In addition, mercurial compounds can also inhibit TrxR by binding to selenol groups. In fact, as a consequence of its softness, mercury has a higher affinity for selenols than thiols (Sugiura et al. 1976; da Conceição Nascimento et al. 2009).

Several in vivo and in vitro studies have suggested that exposure of experimental animals to organic forms of mercury can be accompanied by the induction of oxidative stress (Aschner et al. 2007). The role of TrxR and Trx, at the core of cellular thiol redox control and antioxidant defense (including here the ability to reduce peroxiredoxins; Seo et al. 2000), implicate that inhibition of the Trx system can increase ROS levels. Thus, one of the mechanisms by which MeHg can induce oxidative stress could be via TrxR inhibition. Mahboob et al. (2001) have shown that Hg(II) treatment enhances lipid peroxidation in several tissues, but kidney is one of the most sensitive organs to Hg toxicity. In agreement with this finding, our in vivo results demonstrated that renal TrxR was more inhibited by MeHg than the brain and liver enzymes. Therefore, the inhibition of kidney TrxR may be a new mechanism via which MeHg exerts nephrotoxicity and increases lipid peroxidation (de Freitas et al. 2009).

Herein we have demonstrated a significant inhibition of renal and hepatic TrxR which may be due to a higher deposition of mercury in these organs after MeHg exposure in mice (de Freitas et al. 2009). In addition, the in vitro results indicated that brain TrxR had higher IC_{50} values for the inhibition by MeHg, and consistent with in vivo MeHg exposure, did not cause inhibition of cerebral TrxR, suggesting that brain TrxR is less sensitive to MeHg inhibition after in vivo and in vitro exposure. The absence of cerebral TrxR inhibition after in vivo exposure to MeHg can also be related to a low level of mercury deposition in the brain when compared to liver and kidney. de Freitas et al. (2009) have shown that exposure to MeHg caused accumulation of Hg in the liver, kidney, cerebrum, and cerebellum, but the level of hepatic and renal Hg deposition was approximately 10 times higher than in brain.

The catalytic activity of selenoenzymes, such as TrxR, depends upon the biochemistry of the selenocysteine present at the active sites (Behne et al. 2000). The unique capabilities of the various selenoenzymes, like reduction of hydroperoxides, occur because of the selenocysteine residues. Because the selenol of selenocysteine is ionized at a physiologic pH, it is often more reactive than a cysteine thiol.

Unfortunately, these features that make TrxR so valuable physiologically, also make it more vulnerable to MeHg toxicity (Ralston et al. 2008).

An irreversible inhibitor is one that forms covalent bonds with components of the active site of an enzyme. Because selenocysteine is the principal active site catalytic component of TrxR, MeHg is by definition a highly specific irreversible selenoenzyme inhibitor; MeHg forms covalent bonds between its mercury moiety and the selenium of the selenocysteine of the enzyme (Farina et al. 2009a, b; Ralston et al. 2008). In this case, however, the inhibitor–enzyme complex not only abolished the activity of the inhibited selenoenzyme, it also restricteds selenium release from the MeHg–SeCys complex, severely limiting the bioavailability of selenium for participation in future intracellular cycles of SeCys synthesis (Ralston et al. 2008).

In summary, this study demonstrated, for the first time, that in vivo exposure to MeHg can cause inhibition of liver and kidney TrxR, but not brain TrxR. In vitro exposure caused a dose-dependent inhibition of TrxR, and kidney and liver TrxR were more sensitive to inhibition by MeHg than brain TrxR. Methylmercury can bind to the selenium moiety of selenocysteine, thus directly inhibiting the activity of selenoenzymes (Farina et al. 2009b) and, consequently, disrupting the antioxidant functions and redox state maintained by these enzymes. The results presented here reinforce the central role of selenoproteins in the toxicity of mercurials compounds and strongly indicate that TrxR is an important in vivo molecular target for MeHg.

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