

# Inhibition of the Human Thioredoxin System

## A MOLECULAR MECHANISM OF MERCURY TOXICITY\*<sup>§</sup>

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Mercury toxicity mediated by different forms of mercury is a major health problem; however, the molecular mechanisms underlying toxicity remain elusive. We analyzed the effects of mercuric chloride (HgCl<sub>2</sub>) and monomethylmercury (MeHg) on the proteins of the mammalian thioredoxin system, thioredoxin reductase (TrxR) and thioredoxin (Trx), and of the glutaredoxin system, glutathione reductase (GR) and glutaredoxin (Grx). HgCl<sub>2</sub> and MeHg inhibited recombinant rat TrxR with IC<sub>50</sub> values of 7.2 and 19.7 nM, respectively. Fully reduced human Trx1 bound mercury and lost all five free thiols and activity after incubation with HgCl<sub>2</sub> or MeHg, but only HgCl<sub>2</sub> generated dimers. Mass spectra analysis demonstrated binding of 2.5 mol of Hg<sup>2+</sup> and 5 mol of MeHg<sup>+</sup>/mol of Trx1 with the very strong Hg<sup>2+</sup> complexes involving active site and structural disulfides. Inhibition of both TrxR and Trx activity was observed in HeLa and HEK 293 cells treated with HgCl<sub>2</sub> or MeHg. GR was inhibited by HgCl<sub>2</sub> and MeHg *in vitro*, but no decrease in GR activity was detected in cell extracts treated with mercurials. Human Grx1 showed similar reactivity as Trx1 with both mercurial compounds, with the loss of all free thiols and Grx dimerization in the presence of HgCl<sub>2</sub>, but no inhibition of Grx activity was observed in lysates of HeLa cells exposed to mercury. Overall, mercury inhibition was selective toward the thioredoxin system. In particular, the remarkable potency of the mercury compounds to bind to the selenol-thiol in the active site of TrxR should be a major molecular mechanism of mercury toxicity.

Over centuries, human populations have been exposed to different chemical and physical forms of mercury, all of which have deleterious effects. Past medical applications of mercury included the treatment of syphilis and skin infections, and presently its uses in amalgams and as a preservative in vaccines and topic preparations are very controversial (1). Mercury exists in three oxidation states, Hg<sup>0</sup> (metallic), Hg<sup>+</sup> (mercurous), and Hg<sup>2+</sup> (mercuric), giving rise to distinct inorganic and organic mercurials. Differences in the transport of the different forms

of mercury are responsible for variations in tissue and organ distribution, patterns of biological effect, and toxic potencies (1–4). The kidneys are the primary organs in which inorganic forms of mercury accumulate. Organic mercuric compounds, such as methylmercury (MeHg),<sup>3</sup> being a strong neurotoxin, affect mainly the central nervous system. Other health risks arising from exposure to MeHg include developmental and immunotoxic effects (5, 6). MeHg contamination is a critical public health problem, since fish constitutes the major source of this xenobiotic to humans; in fish, MeHg binds to free amino acids and proteins of muscle tissues and is neither removed nor destroyed by cooking or general food preparation processes. Therefore, MeHg bioaccumulation and concentration through the aquatic food chain puts at risk the human population, who consume fish as part of a daily diet or high end fish predators in excess (4, 7). To date, MeHg is a global pollutant, with elevated levels being detected worldwide (2, 3, 8).

Mercury toxicity has been related to the ability of mercury to form stable complexes with sulfhydryl-containing molecules, such as the cysteine residues of cellular proteins and nonprotein molecules (5, 9–11). GSH, the most abundant intracellular thiol, with 1–10 mM concentrations, is a major antioxidant and redox buffer in human cells. The role of GSH in the molecular mechanism of mercury toxicity has revealed that conjugation of MeHg with GSH contributes to the development of toxicity due to the formation of MeHg-L-cysteine complex (structurally similar to methionine), which by molecular mimicry results in this xenobiotic gaining access across cell membranes through amino acid methionine transporters (see Ref. 5 and references therein). GSH depletion induced by MeHg has also been reported (12). However, to date, GSH involvement is not sufficient to explain the mode of action of mercury in living organisms (13, 14). The possible protective role of selenium against mercury toxicity was first observed by Ganther *et al.* (15). However, the results available currently are still controversial (16–19). Selenides can also be a relevant target for mercurial compounds, and mercury could hypothetically inhibit the biochemical functions involving selenoenzymes (20). Among the 25 human selenoproteins, the well known isoenzymes of glutathione peroxidase (GPx) and mammalian thioredoxin

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<sup>3</sup> The abbreviations used are: MeHg or CH<sub>3</sub>HgCl, methylmercury; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, DL-dithiothreitol; GPx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; HgCl<sub>2</sub>, mercuric chloride; Sec, selenocysteine; Trx, thioredoxin; TrxR, thioredoxin reductase; hTrx, human Trx; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; QTOF, quadrupole time-of-flight.

## Mercury Inhibits the Mammalian Thioredoxin System

reductase (TrxR) are responsible for catalyzing redox reactions involving basic mechanisms in cell growth like DNA synthesis and antioxidant defense. Selenium is incorporated in the form of selenocysteine (Sec) in the active sites of these enzymes and participates in the catalytic mechanism of reduction of peroxide by GSH-dependent GPx and reduction of oxidized thioredoxin (Trx) by NADPH-dependent TrxR (21).

The Trx system is critical for cellular stress response, protein repair, and protection against oxidative damage. Human Trx has a conserved dithiol active site, Cys<sup>32</sup>-Gly-Pro-Cys<sup>35</sup> (22, 23) and, like other mammalian Trx1 proteins, contains three structural cysteine residues (Cys<sup>62</sup>, Cys<sup>69</sup>, and Cys<sup>73</sup>); these additional residues make Trx1 susceptible to oxidation via generation of a second disulfide (Cys<sup>62</sup>-Cys<sup>69</sup>) which leads to loss of catalytic activity (22–24). Mammalian TrxR has a broad range of functions, which include reducing hydroperoxides and regenerating compounds, such as dehydroascorbate, lipoate, and ubiquinone. Additionally, selenium-compromised forms of TrxR induce apoptosis (25). Both TrxR and Trx are widely distributed in different mammalian organs and tissues (26). Among cell types, epithelial, secretory, blood, and neuronal cells showed high immunoreactivity by immunohistochemical location (26). It has also been demonstrated that Trx and TrxR are synthesized in nerve cell bodies and then transported to synaptic terminals, where they are likely to be involved in synaptic transmission (27). Biomarkers have been used to assess exposure and disease risks of human populations. Until now, hair and blood mercury concentrations are considered the only valid biomarkers of human exposure to MeHg (1, 7, 28), although quantification of exposure to MeHg gives limited information about the toxic effects exerted on the organism. In fact, depending on the diet, state of chemoprotection, and other specific characteristics of susceptibility of an individual, food-associated chemicals, such as MeHg, can have a certain degree of negative impact (29, 30). Therefore, identification of early alterations in living organisms that anticipate processes of serious disruption by xenobiotics or diseases is crucial for the protection of human populations.

Cellular responses to a specific hazardous food chemical may reveal whether the body contains the chemical and how the toxic effects are developing. On the basis that mercury has a chemical propensity to interact with thiols and selenols, we hypothesized that thiol redox systems and the associated selenoenzymes might be involved in the development of mercury toxicity. In this study, we have investigated the effects of inorganic mercuric chloride (HgCl<sub>2</sub>) and organic methylated monomethylmercury (CH<sub>3</sub>HgCl, herein referred to as MeHg) on components of the Trx system, TrxR and Trx, and their related components in the glutaredoxin system, glutathione reductase (GR) and glutaredoxin (Grx). The main goal is the investigation of the molecular mechanisms accounting for mercury toxicity as well as the identification of a potential biomarker indicative of mercury effects.

### EXPERIMENTAL PROCEDURES

**Materials and Enzymes**—The preparation of recombinant rat TrxR was carried out as previously described (31). The protein had 47% specific activity of wild-type TrxR as determined

by the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay; results are reported with respect to active enzyme. Preparations of wild-type and double mutant C62S/C73S human Trx were as previously published (32). His-tagged human Grx1 protein was purified as described before (33). Yeast GR, GSSG, insulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydroxyl-ethyl disulfide, NADPH, DL-dithiothreitol (DTT), GSH, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), mercuric chloride (HgCl<sub>2</sub>), and monomethylmercury chloride (MeHg) were purchased from Sigma. HgCl<sub>2</sub> and MeHg were prepared as 100 mM stocks in MilliQ water and DMSO, respectively. Tris base was supplied by Calbiochem and was of molecular biological grade with 99.2% purity to titration and <0.0005% heavy metals.

**Solutions and Reagent Preparation**—For *in vitro* assays performed with recombinant proteins, buffers and enzyme solutions were processed to ensure the exclusion of metal ion chelator EDTA and to minimize the presence of traces of other metal ions that could have interfering effects on TrxR activity. Stocks of purified TrxR and Trx were stored in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE buffer). To remove EDTA, stock protein solutions were loaded onto Sephadex G-25-containing PD-10 columns (Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl, pH 7.5 (Tris buffer). The protein concentration of eluted protein solution was then determined. The stock of purified Grx was stored in phosphate-buffered saline, pH 7.4, and was therefore used directly in incubation assays with mercurial compounds. To minimize traces of metal ions, buffers used were prepared with fresh MilliQ water ( $\Omega = 18.2$ ), followed by 1-h orbital agitation with Chelex<sup>®</sup> 100 chelating ion exchange resin (5% (w/v); Bio-Rad). The buffer was then recovered by filtration (0.2- $\mu$ m filter). For assays involving enzyme activity determination in cell lysates, buffers and enzyme solutions were not subjected to the procedures mentioned above, since the metal ions and salts present would be negligible relative to that contained in the collected lysates.

**TrxR Activity Determination by DTNB Reduction Assay**—In 96-well plates, 50 nM recombinant rat TrxR (EDTA-free) was preincubated with HgCl<sub>2</sub> or MeHg of different concentrations and 200  $\mu$ M NADPH in 50 mM Tris-HCl, pH 7.5 (final volume 100  $\mu$ l) for various times at room temperature. Then TrxR activity was determined as previously described (34) upon adding 100  $\mu$ l of Tris buffer containing DTNB and NADPH (final concentration 5 mM and 200  $\mu$ M, respectively). Briefly, the absorbance at 412 nm was followed using a VERSA microplate reader (Molecular Devices), and TrxR activity was obtained as the linear change in absorbance over the initial 2 min.

**Trx System Activity Determination by Insulin Reduction Assay**—Activity of the Trx system was determined by modification of a method previously described (34). In disposable semimicro-UV cuvettes (Plastibrand), 12 nM recombinant rat TrxR (EDTA-free) and 3  $\mu$ M hTrx were preincubated with HgCl<sub>2</sub> or MeHg of different concentrations and 200  $\mu$ M NADPH in 50 mM Tris-HCl, pH 7.5 (final volume 500  $\mu$ l) for 10 min at room temperature. Then 100  $\mu$ l of Tris buffer containing insulin and NADPH (final concentration 160 and 200  $\mu$ M, respectively) was added, and absorbance at 340 nm was followed in an Ultrospec 3000 spectrophotometer. Activity

was calculated as the linear change in absorbance over the initial 5 min.

**Determination of Free Thiols Content in Trx and Grx with DTNB**—The number of free thiols in reduced Trx and Grx after treatment with  $\text{HgCl}_2$  or MeHg was determined by the method of Ellman with modifications (35). Briefly, fully reduced recombinant human Trx and Grx were prepared by incubating the proteins with 10 mM DTT at 37 °C for 15 min, followed by passing the protein solution through a NAP-5 desalting column to remove excess DTT. The concentration of eluted protein was calculated from absorbance measurements at 280 and 310 nm.  $\text{HgCl}_2$  was incubated at different molar ratios with 10  $\mu\text{M}$  fully reduced Trx or Grx at room temperature. After incubation, 1 mM DTNB and 6 M guanidine HCl in 0.2 M Tris-HCl, pH 8, was added (final volume 500  $\mu\text{l}$ ). The absorbance was read at 412 nm against a reference cuvette that contained the same amount of DTNB without protein, and the number of free thiols was calculated using a molar extinction coefficient of 13,600  $\text{M}^{-1} \text{cm}^{-1}$ .

**Gel Electrophoresis**—Untreated and mercurial compound-treated Trx and Grx proteins (10  $\mu\text{g}$ ) were loaded onto 12% bis-Tris NuPAGE® gel (Invitrogen) and separated using MES running buffer. The gel was then Coomassie-stained.

**Mass Spectra Analysis**—Human Trx was DTT-treated to obtain the fully reduced form. Following desalting to remove excess DTT, the protein was treated with  $\text{HgCl}_2$  or MeHg, and excess mercurial compound was removed by another desalting process. These samples were divided into two aliquots, and 500  $\mu\text{l}$  were further DTT-treated as an attempt to obtain the fully reduced form of the protein again; excess DTT was also removed after treatment. Prior to mass spectra analysis, all samples were passed through NAP-5 columns pre-equilibrated with MilliQ water. The buffer-free solutions were diluted to a final protein concentration of  $\sim 10 \mu\text{M}$ . Data were acquired on a QTOF Ultima API spectrometer (Waters Corp., Milford, MA) equipped with a standard Z-spray API source in positive mode (at the Protein Analysis Center of Karolinska Institute). Samples were introduced from the metal-coated borosilicate glass capillary needle (Proxeon Biosystems A/S, Odense, Denmark), and the capillary voltage was 1.5 kV. The instrument was calibrated between 300 and 1400  $m/z$  with myoglobin before the run. The data were sampled over a mass range between 300 and 3000  $m/z$  with a scan of 1 s for about 5 min. The spectra were combined and deconvoluted to zero charged ions (average mass) using Maxlynx 4.0 software (Waters Corp., Milford, MA).

**Cell Culture**—Human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium and human HEK 293 embryonic kidney cells in RPMI medium. Both media were supplemented with glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were incubated at 37 °C in humidified air containing 5%  $\text{CO}_2$ .

**Cell Viability Assay**—Cells were seeded into 96-well plates at a density of  $3 \times 10^6$  cells/well and allowed to attach for 24 h before the addition of different concentrations of  $\text{HgCl}_2$  and MeHg. Cell viability at the time of drug addition (time 0) and following 24, 48, and 72 h of drug treatment were determined by the MTT assay. MTT was added to each well (final concen-

tration 400  $\mu\text{g}/\text{ml}$ ), and plates were incubated at 37 °C for 4 h to allow viable cells to metabolize MTT. Medium in each well was then aspirated, and formazan formed by viable cell dehydrogenase-mediated reduction of MTT was dissolved by the addition of DMSO/glycine buffer (pH 10.5) (ratio 4:1). Cell viability, which was proportional to the amount of formazan formed, was determined by measuring the absorbance at 550 nm.

**Preparation of Cell Lysates**—Cells ( $1 \times 10^6$ ) were plated onto 100-mm plates, and when cell confluence reached  $\sim 70\%$ , cells were incubated with a fresh volume of medium containing  $\text{HgCl}_2$  and MeHg of different concentrations for 7, 15, and 24 h. Whole cell lysates of control and mercurial compound-treated cells were obtained by lysing cell pellets (composed of attached and floating cells) in lysis buffer (25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 20 mM NaF, 1 mM sodium orthovanadate, 20 mM sodium  $\beta$ -glycerophosphate, 10 mM sodium pyrophosphate, 0.5% Triton X-100 containing freshly added protease inhibitor mixture) (Roche Applied Science). Before use, lysates were precleared by centrifugation at 13,000 rpm for 6 min, followed by protein content determination using a modified Bradford assay (Bio-Rad) according to the manufacturer's manual.

**Enzyme Activity Determination in Cell Lysates**—TrxR and Trx activity in cell lysates were measured in 96-well plates using an end point insulin assay (36). For TrxR activity measurement, 25  $\mu\text{g}$  (HeLa) or 40  $\mu\text{g}$  (HEK 293) of protein cell lysate was incubated in a final volume of 50  $\mu\text{l}$  containing 0.3 mM insulin, 660  $\mu\text{M}$  NADPH, 2.5 mM EDTA, and 5  $\mu\text{M}$  human C62S/C73S Trx in 85 mM HEPES, pH 7.6, for 20 min at room temperature. Control wells containing incubate of cell lysates with similar reagents excluding human Trx were set up too. Then 250  $\mu\text{l}$  of 1 mM DTNB and 240  $\mu\text{M}$  NADPH in 6 M guanidine hydrochloride, 200 mM Tris-HCl, pH 8, was added, and absorbance readings were taken at 412 nm. TrxR activity was represented as the  $A_{412 \text{ nm}}$  by subtracting that in the control well. For determination of Trx activity, procedures were the same as for the TrxR activity assay; cell lysates were incubated in reaction solutions with 600 nM recombinant rat TrxR except for control wells.

Measurements of GR activity in cell lysates were carried out in 96-well plates using published methods (37, 38) with modifications. Briefly, to 25  $\mu\text{g}$  of protein cell lysate per well (volume made up to 80  $\mu\text{l}$  with 100 mM phosphate buffer, pH 7), 120  $\mu\text{l}$  of GSSG and NADPH in phosphate buffer (final concentration 1 mM and 200  $\mu\text{M}$ , respectively) was added, and absorbance at 340 nm was followed. GR activity was obtained as the linear change in absorbance over the initial 10 min. Grx activity in cell lysates was measured using the same assay as described for detecting activity in purified Grx protein (see below); Grx1 protein sample was replaced by 100  $\mu\text{g}$  of protein cell lysate.

**Determination of GR Activity**—In cuvettes,  $\text{HgCl}_2$  or MeHg of different concentrations was added to 2 nM yeast GR and 200  $\mu\text{M}$  NADPH and incubated for 5 min at room temperature. Upon mixing 100 mM Tris-HCl, pH 7.4, containing GSSG (final concentration 1 mM) to a final volume of 500  $\mu\text{l}$ , GR activity was determined by measuring the decrease in absorbance at 340 nm during the initial 3 min using an Ultrospec 3000 spectrophotometer.

## Mercury Inhibits the Mammalian Thioredoxin System

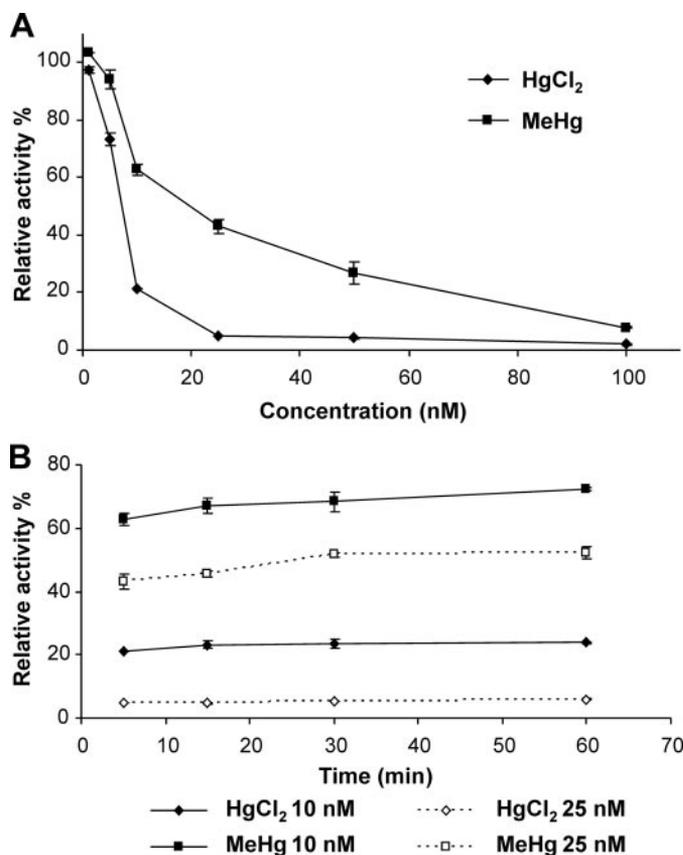
**Determination of Grx Activity**—HgCl<sub>2</sub> or MeHg of different concentrations was incubated with 100 μM fully reduced recombinant human Grx1 (DTT-treated and desalted) for 15 min at room temperature, and excess mercurial compounds were removed by passing the protein samples through NAP-5 columns. The enzymatic activity of Grx1 was determined as previously described (39, 40). Briefly, in a 96-well plate, substrate hydroxyl-ethyl disulfide (0.7 mM) was mixed with 0.1 mg/ml bovine serum albumin, 1 mM GSH, 200 μM NADPH, and 6 μg/ml yeast GR in 100 mM Tris-HCl, pH 8, 2 mM EDTA. After 3 min of incubation at 30 °C, different concentrations (ranging from 0 to 50 nM) of untreated or mercury-treated Grx1 (desalted) were added to start the reactions. The rates of decrease at 340 nm were recorded against a reference sample containing similar reagents excluding Grx; results were plotted against Grx1 concentrations to provide a linear curve. The effect of mercurial compounds on Grx activity was determined by comparing the slopes of curves obtained from mercury-treated and untreated protein samples.

### RESULTS

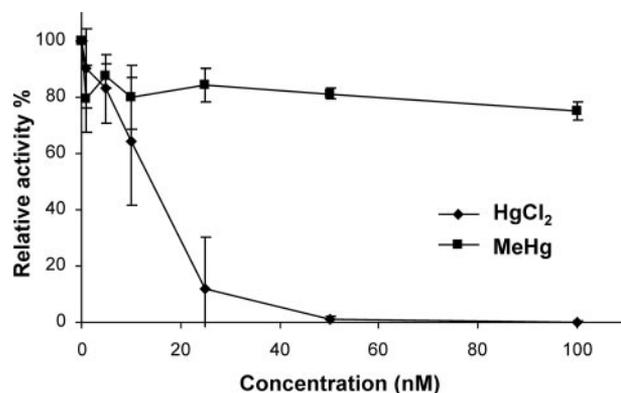
**Effects of Mercurial Compounds on Mammalian TrxR Activity**—The effect of inorganic HgCl<sub>2</sub> and organic MeHg on purified mammalian TrxR was investigated. Different concentrations of HgCl<sub>2</sub> and MeHg were incubated with NADPH-reduced and EDTA-free rat TrxR protein (50 nM) for between 5 and 60 min at room temperature, and TrxR activity was determined using the DTNB reduction assay. As shown in Fig. 1, A and B, both mercurial compounds inhibited enzyme activity in a dose-dependent manner as early as within 5 min of mercury exposure. Inorganic HgCl<sub>2</sub> exhibited greater inhibitory effect, with an IC<sub>50</sub> value of 7.2 nM, as compared with organic MeHg, showing an IC<sub>50</sub> of 19.7 nM. Inhibitory experiments were also performed in the presence of the metal ion chelator EDTA at 1 mM; results showed similar dose dependence as seen in assays carried out in the absence of EDTA; higher IC<sub>50</sub> values of 14.2 and 25.6 nM for HgCl<sub>2</sub> and MeHg, respectively, were obtained (data not shown). Inhibitory effect of these mercurial compounds on mammalian TrxR was observed to be rapid and not time-dependent (Fig. 1B).

**Effects of Mercurial Compounds on Trx Activity and Structure**—To study the effect of mercurial compounds on the whole Trx system, HgCl<sub>2</sub> and MeHg were preincubated at different concentrations with components of the Trx system for 10 min at room temperature, followed by measurement of Trx system activity using the insulin reduction assay. Results showed that HgCl<sub>2</sub> brought about a similar extent of inhibition of TrxR/Trx-mediated disulfide reduction with comparable IC<sub>50</sub> values (Fig. 2) as that achieved for TrxR inhibition (Fig. 1A). Interestingly, across the concentrations tested, MeHg failed to exhibit a significant extent of Trx inhibition (Fig. 2), which was in contrast to an MeHg-mediated drastic drop in TrxR activity as determined by the DTNB reduction assay (Fig. 1A). This could be due to MeHg redistribution, since it may also interact with the Trx, present in large excess, or with insulin.

The next objective was to investigate effects on the Trx protein (independent of TrxR), and for that we employed free thiol determinations. In the assay, the mercurial compounds were

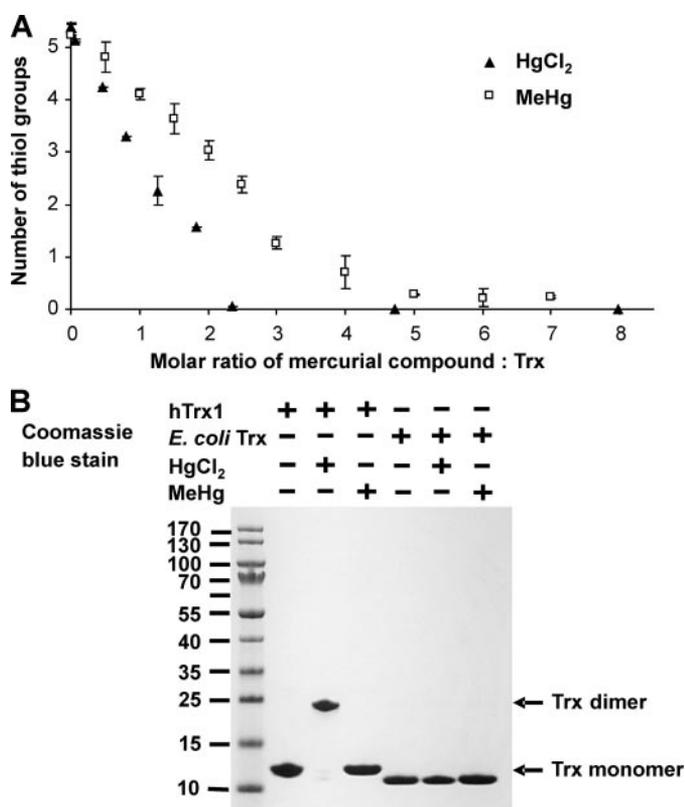


**FIGURE 1. Inhibition of mammalian TrxR by inorganic (HgCl<sub>2</sub>) and organic (MeHg) mercurial compounds *in vitro*.** A, concentration-dependent inhibition of 50 nM recombinant rat TrxR (EDTA-free) by different concentrations of HgCl<sub>2</sub> and MeHg after a 5-min preincubation. B, the effect of HgCl<sub>2</sub> and MeHg on the activity of 50 nM recombinant TrxR (EDTA-free) was investigated at different time points. TrxR activity was determined in the presence of 200 μM NADPH by a DTNB reduction assay (see "Experimental Procedures"), and relative activity was expressed as a percentage of untreated control. All data points are means ± S.D. of two independent experiments.

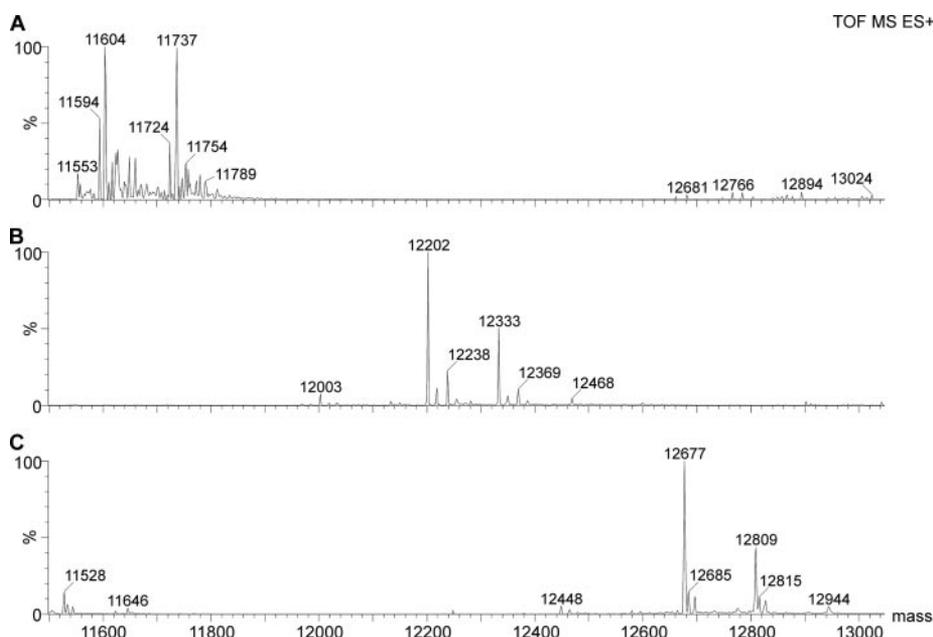


**FIGURE 2. Inhibition of mammalian Trx system by inorganic (HgCl<sub>2</sub>) and organic (MeHg) mercurial compounds *in vitro*.** 12 nM recombinant rat TrxR (EDTA-free) and 3 μM hTrx were incubated with 200 μM NADPH and HgCl<sub>2</sub> or MeHg at concentrations as indicated for 10 min. Activity was determined by an insulin reduction assay (see "Experimental Procedures"), where A<sub>340 nm</sub> was followed upon adding insulin (final concentration 200 μM). Relative activity was expressed as a percentage of untreated control. All data points are means ± S.D. of three independent experiments.

incubated with 10 μM human Trx fully reduced by DTT. Both HgCl<sub>2</sub> and MeHg caused an apparent oxidizing effect on the redox state of hTrx that was independent of time and fast, since effects could be detected in 30 s (results not shown). The



**FIGURE 3. Effect of HgCl<sub>2</sub> and MeHg on thiol groups in Trx.** *A*, recombinant human Trx was fully reduced by DTT, and excess DTT was removed by desalting over a NAP-5 column. HgCl<sub>2</sub> or MeHg was incubated with a 10 μM concentration of the reduced protein at varying molar ratios as indicated. Remaining free thiol groups were then determined by titration with DTNB. All data points are means ± S.D. of two independent experiments. *B*, HgCl<sub>2</sub> and MeHg were incubated with 10 μM reduced hTrx1 or *E. coli* Trx at a molar ratio of 2.5 and 5, respectively. Protein samples were loaded onto a bis-Tris gel and separated by SDS-PAGE, and the gel was Coomassie-stained. HgCl<sub>2</sub>-treated hTrx appeared as a dimer while MeHg-treated hTrx stayed as a monomer. The *E. coli* Trx-treated samples remained in the monomeric form independent of the mercurial compound used.



**FIGURE 4. Mass spectra of hTrx samples attained by QTOF mass spectrometry electrospray analysis.** Human Trx1 protein was treated as described under "Experimental Procedures." The protein samples were desalted of excess mercurial compounds and buffers, and final protein solutions of ~10 μM in MilliQ water were electrosprayed into the QTOF spectrometer for analysis. *A*, spectra of the fully reduced protein; *B*, HgCl<sub>2</sub>-treated hTrx; *C*, MeHg-treated hTrx.

increase of the molar ratio of mercurial compounds to hTrx protein ( $I/Trx$ ) had a progressive effect, and a ratio of  $\geq 2.5$  for HgCl<sub>2</sub> and  $\geq 5$  for MeHg resulted in complete loss of the five free thiols (Fig. 3A).

The hTrx protein samples treated with mercurial compounds at doses that fully oxidized thiol groups were subjected to electrophoresis on a bis-Tris nonreducing gel and Coomassie-stained. The HgCl<sub>2</sub>-treated sample appeared predominantly as a protein band of around 24 kDa, versus the untreated sample occupying the position corresponding to 12 kDa (Fig. 3B), implying that HgCl<sub>2</sub> treatment promoted Trx dimerization, which was in agreement with gel filtration HPLC analysis (data not shown). Nevertheless, HgCl<sub>2</sub> failed to bring about dimerization of *Escherichia coli* Trx that only possesses the active site cysteine residues, Cys<sup>32</sup> and Cys<sup>35</sup>, and lacks structural cysteines (Fig. 3B). MeHg-treated hTrx as well as MeHg-treated *E. coli* Trx appeared as monomeric bands at 12 kDa (Fig. 3B). Direct interaction of mercury with hTrx was confirmed by doing mass spectrometry analysis of hTrx treated with HgCl<sub>2</sub> and MeHg (excess mercurial compounds removed by desalting). Compared with the spectrum of untreated fully reduced hTrx (mass peaks 11,604 and 11,737; Fig. 4A), the spectrum of HgCl<sub>2</sub>-treated samples (mass peaks 12,202 and 12,333; Fig. 4B) clearly showed the increase in mass of  $597 \pm 1$  Da, which corresponds to the formation of adducts with the presence of three mercury atoms (see Table 1). Although results from gel electrophoresis and HPLC analysis showed hTrx dimerization in the presence of HgCl<sub>2</sub>, the peak corresponding to the sample dimers was not significant for the total content, which could be a consequence of sample degradation during analysis. In MeHg-treated samples, the spectrum (Fig. 4C) showed predominant mass peaks at 12,677 and 12,809 with a mass increase of 1073 Da, which points to the presence of five CH<sub>3</sub>Hg<sup>+</sup> groups (Table 1). The subsequent addition of excess DTT to the mercury-treated samples failed to displace mercury completely from the hTrx protein. This was particularly evident for HgCl<sub>2</sub>-DTT-treated samples, where the most abundant peaks of hTrx still contained one or two mercury atoms (Table 1; spectra not shown). The results agreed with the gel electrophoresis, where no dimerization was detected (data not shown) as well as with the determination of 2–3 free thiols in HgCl<sub>2</sub>-DTT-treated hTrx samples (Table 1).

*Effects of Mercurial Compounds on TrxR and Trx in Cells*—To assess effects of mercury treatment on the thioredoxin system, the HeLa cell line, which has a high TrxR and Trx expression, was selected as a model. Subsequent studies were also carried out in HEK 293 cells. The effect of HgCl<sub>2</sub> and MeHg on the growth of HeLa and HEK cell lines was eval-

TABLE 1

Structural and biological effects of HgCl<sub>2</sub> and MeHg on hTrx

Mass spectra analysis of approximately 10 μM hTrx samples was performed by time-of-flight mass spectrometry electrospray with S.D. of ±1 Da. The most abundant proteins are indicated in boldface type.

Protein	-SH	Activity in insulin assay	Mass spectra data		
			Mass	Mass difference	Comments
Fully reduced hTrx	5	100%	<b>11,604 (A)<sup>a</sup>; 11737 (B)<sup>a</sup></b>		
HgCl <sub>2</sub> -treated hTrx ( <i>I/Trx</i> = 2.5)	0	No activity	(12,003)	+399 (A)	2 × Hg 200.59 - 4H = 397.18
			<b>12,202</b>	<b>+598 (A)</b>	<b>3 × Hg 200.59 - 4H = 597.8</b>
			12,238	+634 (A)	3 × Hg 200.59 - 4H + 1 × Cl 35.45 = 633.22
			<b>12,333</b>	<b>+596 (B)</b>	<b>3 × Hg 200.59 - 4H = 597.8</b>
			12,369	+632 (B)	3 × Hg 200.59 - 4H + 1 × Cl 35.45 = 633.22
HgCl <sub>2</sub> -treated hTrx + DTT reduction	2-3	No activity	(11,606)	0 (A)	
			(11,737)	0 (B)	
			<b>11,805</b>	<b>+201 (A)</b>	<b>1 × Hg 200.59 - 2H = 198.59</b>
			<b>11,936</b>	<b>+199 (B)</b>	<b>1 × Hg 200.59 - 2H = 198.59</b>
			12,003	+399 (A)	2 × Hg 200.59 - 4H = 397.18
			12,135	+398 (B)	2 × Hg 200.59 - 4H = 397.18
CH <sub>3</sub> HgCl-treated hTrx ( <i>I/Trx</i> = 5)	0	No activity	<b>12,677</b>	<b>+1073 (A)</b>	<b>5 × CH<sub>3</sub>-Hg<sup>+</sup> 215.59 - 5H = 1073</b>
			<b>12,809</b>	<b>+1072 (B)</b>	<b>5 × CH<sub>3</sub>-Hg<sup>+</sup> 215.59 - 5H = 1073</b>
CH <sub>3</sub> HgCl-treated hTrx + DTT reduction	5	~50%	<b>11,606</b>	0 (A)	
			11,737	0 (B)	
			11,821	+215 (A)	1 × CH <sub>3</sub> -Hg <sup>+</sup> 215.59 - 1H = 214.59
			11,952	+215 (B)	1 × CH <sub>3</sub> -Hg <sup>+</sup> 215.59 - 1H = 214.59

<sup>a</sup> Forms A and B coexist in the preparation of recombinant expressed hTrx and differ by the presence of the N-terminal amino acid methionine with a molecular mass of 133 Da.

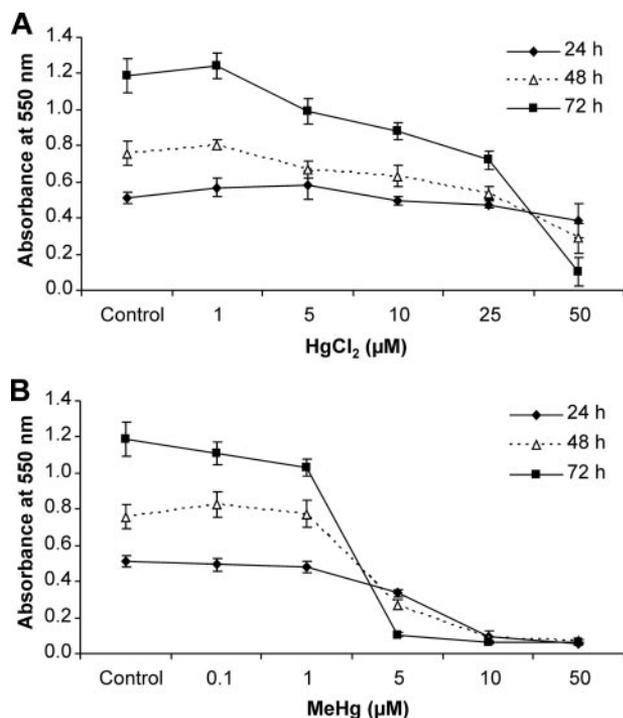


FIGURE 5. Cell growth and toxicity profile of mercurial compounds. HeLa cells were exposed to HgCl<sub>2</sub> (A) and MeHg (B) at different concentrations for time points as indicated prior to viability determination by MTT assay. Data points are means of at least 4-well readings on a single experiment. Assays were performed on three separate experiments.

uated using the MTT cell viability assay. HgCl<sub>2</sub> and MeHg exerted a concentration-dependent growth inhibition of HeLa cells with respective GI<sub>50</sub> values of 20.6 and 2.9 μM after 72 h of exposure (Fig. 5, A and B, and Table 2). For the same time exposure, HEK 293 cells displayed GI<sub>50</sub> values of 6.0 and 2.8 μM (72 h), respectively, for HgCl<sub>2</sub> and MeHg (Table 2). The results were in agreement with previous work (41–44) and with reported values of lethal doses (LD<sub>50</sub>) (2), showing that organic MeHg is generally the more toxic form for living organisms. Furthermore, cell viability assays conducted for shorter time

exposure at 24 and 48 h showed GI<sub>50</sub> values comparable with those for 72-h treatment (Fig. 5B and Table 2), although the cytotoxic effect of HgCl<sub>2</sub> on HeLa cells was generally delayed, being apparent between 24 and 48 h following treatment (Fig. 5A).

TrxR and Trx activities were determined in cell lysates using the end point insulin reduction assay. Both HgCl<sub>2</sub> and MeHg caused a dose- and time-dependent inhibition of TrxR activity (Fig. 6, A and B). A decrease in TrxR activity in HeLa cells was evident within only 7 h of mercury exposure, when microscopic observations showed no morphological changes indicative of cell death (results not shown). Both mercurial compounds also dose-dependently caused a decrease in Trx activity (Fig. 7, A and B). As compared with TrxR inhibition, the extent of inhibitory effect of HgCl<sub>2</sub> and MeHg on Trx activity of HeLa cells was less significant and remained relatively unchanged at the time points investigated (Fig. 7, A and B, and Table 2). These results indicate that in a cellular system, mercurial compounds inhibit both TrxR and Trx, with TrxR being the preferential target. Studies performed with HEK 293 cells showed a similar trend of TrxR and Trx inhibition by mercurial compounds (Table 2 and supplemental Figs. 1 and 2).

In general, TrxR and Trx inhibition in HeLa and HEK cells were correlated to cytotoxicity exerted by these mercurial compounds (Table 2). Interestingly, although an *in vitro* assay with recombinant mammalian TrxR indicated that inorganic HgCl<sub>2</sub> was the more potent inhibitor, results obtained from the cell-based assays had shown otherwise (Table 2). Organic MeHg was found to be more growth-inhibiting, and at equivalent concentrations, the extent of MeHg-mediated TrxR and Trx inhibition was more profound. The opposing orders of inhibitory potencies for ionizable HgCl<sub>2</sub> on recombinant protein and in cells could be due to the free Hg<sup>2+</sup> ions being chelated or complexed in the cell culture medium such that the effective inhibiting dose made available intracellularly was reduced. The higher bioavailability of MeHg in cells, and thus higher TrxR and Trx inhibition at equivalent doses, accounted at least in

TABLE 2

Effects of mercury compounds on cell viability and growth of HeLa and HEK 293 cells and their inhibitory effects on TrxR and Trx

Compound	<i>In vitro</i> inhibition of TrxR IC <sub>50</sub> (5 min) <sup>a</sup>	Cell line	Cell-based assays								
			Cell growth inhibition evaluated for various time exposures GI <sub>50</sub> <sup>b</sup>			TrxR activity in lysates of cells exposed to mercury treatment at various time points IC <sub>50</sub> <sup>b</sup>			Trx activity in lysates of cells exposed to mercury treatment at various time points IC <sub>50</sub> <sup>b</sup>		
			24 h	48 h	72 h	7 h	15 h	24 h	7 h	15 h	24 h
	<i>nM</i>			$\mu\text{M}$		$\mu\text{M}$		$\mu\text{M}$			
HgCl <sub>2</sub>	7.2 ± 0.1	HeLa	ND <sup>c</sup>	15.5 ± 4.1	20.6 ± 2.1	12.0 ± 4.1	8.2 ± 0.3	5.4 ± 0.9	29.9 ± 6.4	35.9 ± 3.7	32.0 ± 7.8
HgCl <sub>2</sub>	7.2 ± 0.1	HEK 293	6.5 ± 1.08	5.9 ± 0.96	6.0 ± 0.36	5.0 ± 1.3	5.0 ± 1.3	20.1 ± 5.4			
MeHg	19.7 ± 0.7	HeLa	2.4 ± 0.9	2.5 ± 1.1	2.9 ± 1.7	6.5 ± 2.9	4.7 ± 0.1	1.4 ± 0.3	8.1 ± 3.1	9.8 ± 2.0	6.7 ± 1.3
MeHg	19.7 ± 0.7	HEK 293	2.5 ± 0.8	2.4 ± 0.5	2.8 ± 0.9		3.2 ± 1.3			3.7 ± 1.9	

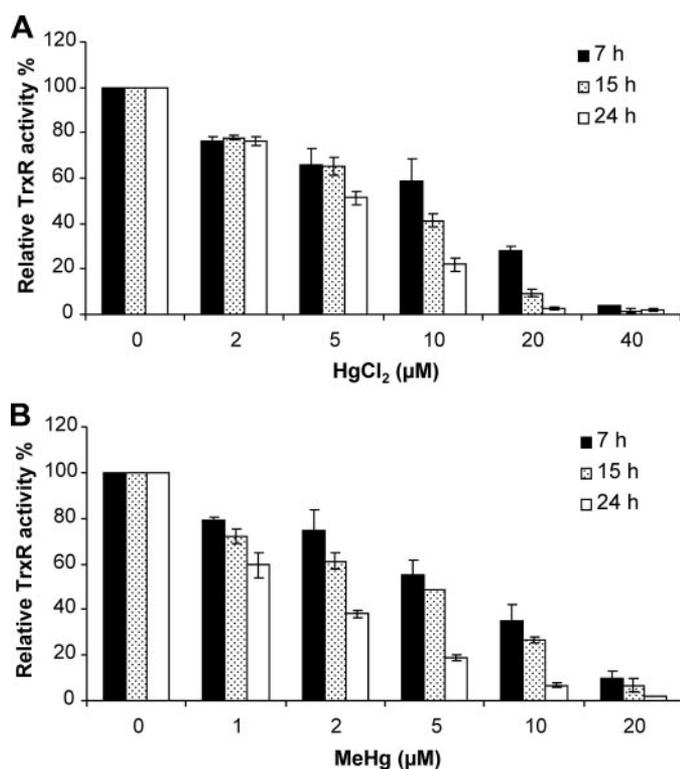
<sup>a</sup> IC<sub>50</sub> (concentration to inhibit 50% of enzyme activity) represents the mean of two independent experiments.<sup>b</sup> GI<sub>50</sub> (concentration to inhibit 50% cell growth) and IC<sub>50</sub> values represent the means of three independent experiments.<sup>c</sup> Not detected.

FIGURE 6. Effects of mercurial compounds on TrxR activity in HeLa cells. Cells were exposed to HgCl<sub>2</sub> (A) and MeHg (B) at different concentrations as indicated for 7, 15, and 24 h, and cell lysates were collected for TrxR activity measurement using the end point insulin reduction assay (see "Experimental Procedures"). TrxR activity was expressed as a percentage of untreated control. All data points are means ± S.E. of three independent experiments.

part for its greater cytotoxicity. Nonetheless, we also acknowledge the probable involvement of other mechanistic targets through which MeHg exerts its cytotoxic effect. For instance, MeHg is reported to interact with the GSH system (5, 6, 12, 13, 45, 46).

**Effects of Mercurial Compounds on GR Activity in Vitro and in Cells**—The effect of mercurial compounds on GR, a member of the glutathione-glutaredoxin system and a structural homolog of mammalian TrxR, was also investigated. NADPH-reduced GR protein (2 nM) was preincubated for 5 min with HgCl<sub>2</sub> or MeHg over a range of concentrations previously shown to bring about enzyme inhibition of rat TrxR (Fig. 1A). A dose-dependent inhibition of GR was observed, with HgCl<sub>2</sub> exhibiting a stronger inhibitory effect with an IC<sub>50</sub> value of 2.2

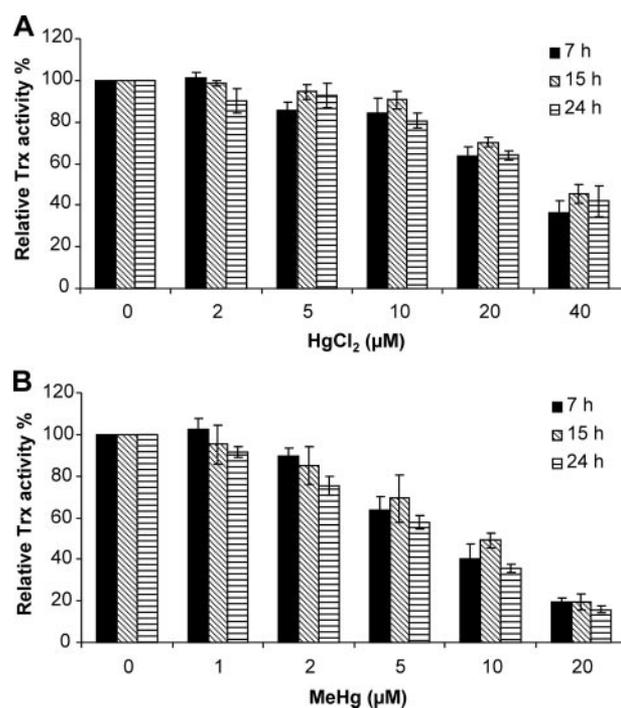
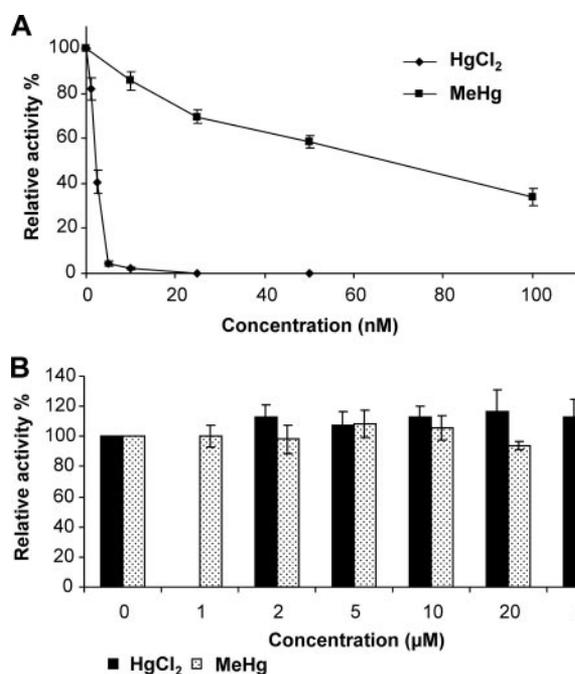


FIGURE 7. Effects of mercurial compounds on Trx activity in HeLa cells. Cells were exposed to HgCl<sub>2</sub> (A) and MeHg (B) at different concentrations as indicated for 7, 15, and 24 h, and cell lysates were collected for Trx activity measurement using the end point insulin reduction assay (see "Experimental Procedures"). Trx activity was expressed as a percentage of untreated control. All data points are means ± S.E. of three independent experiments.

nM as compared with MeHg with an IC<sub>50</sub> value of 67.3 nM (Fig. 8A). We next tested the GR activity in cell lysates of HeLa cells exposed to 24-h treatment of various concentrations of mercurial compounds. Results showed that both HgCl<sub>2</sub> and MeHg failed to bring about a significant effect on the activity of cellular GR (Fig. 8B) when compared with the dramatic reduction in cellular TrxR activity caused by the same concentrations of mercurial compounds (Fig. 6, A and B).

**Effects of Mercurial Compounds on Grx Activity in Vitro and in Cells**—The effect of mercurial compounds on the GSH-dependent oxidoreductase Grx1, a member of the Trx superfamily of proteins with the Cys-Pro-Tyr-Cys active site was examined. In an *in vitro* experiment, 100 μM fully reduced Grx1 was treated with 50 and 150 μM HgCl<sub>2</sub>. The protein sample exposed to the higher HgCl<sub>2</sub> dose completely precipitated, and activity could not be detected. To the other sample, upon removal of



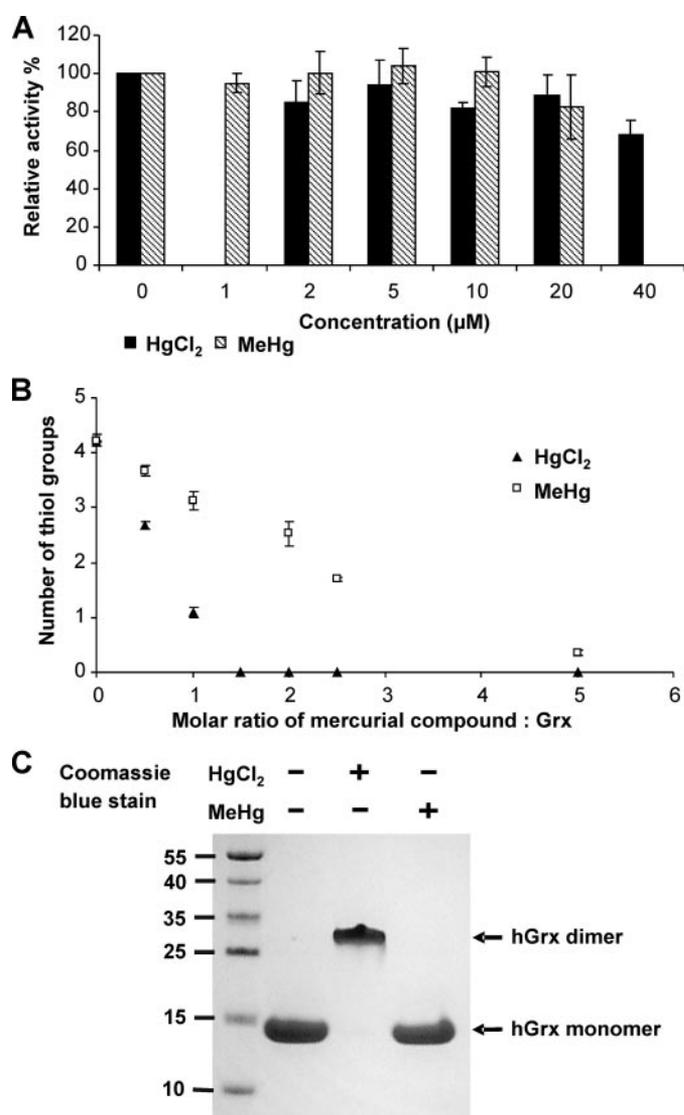
**FIGURE 8. Effects of mercurial compounds on GR activity in HeLa cells.** *A*, concentration-dependent *in vitro* inhibition of 2 nM GR by different concentrations of HgCl<sub>2</sub> and MeHg after a 5-min preincubation. *B*, cells were exposed to HgCl<sub>2</sub> or MeHg at different concentrations as indicated for 24 h, and cell lysates were collected for GR activity measurement. GR activity was expressed as a percentage of untreated control. All data points are means  $\pm$  S.D. of three independent experiments.

excess HgCl<sub>2</sub> using a desalting column, Grx activity determination by the hydroxyl-ethyl disulfide assay showed that only 22% of activity was retained. A similar experiment was performed with 200 and 500  $\mu$ M MeHg; no precipitation occurred, which enabled subsequent Grx activity determination that showed activity reductions to 54 and 48%, respectively. The effects of HgCl<sub>2</sub> and MeHg on Grx activity in intact cells were also determined. As shown in Fig. 9*A*, in general, mercury treatment for 24 h did not lead to a significant decrease in Grx activity of HeLa cells. These results are in contrast to a marked inhibition of Trx activity brought about by the same doses of mercurial compounds (Fig. 7, *A* and *B*).

The reactivity of HgCl<sub>2</sub> and MeHg with thiol groups in the reduced form of Grx1 with five cysteines was also investigated. Consistent with results obtained for Trx (Fig. 3, *A* and *B*), both mercurial compounds caused a dose-dependent oxidation of cysteine residues, with HgCl<sub>2</sub> being more reactive, since a molar ratio of  $I/Grx \geq 1.5$  led to a complete loss of free thiols (Fig. 9*B*). Moreover, a Coomassie-stained bis-Tris nonreducing gel containing Grx protein samples treated with mercurial compounds at doses that fully oxidized thiol groups showed that HgCl<sub>2</sub> treatment caused Grx1 dimerization (Fig. 9*C*).

## DISCUSSION

The chemical propensity of mercury for sulfhydryl groups has long been known. Interaction of mercury species with thiol-containing molecules plays a key role in mechanisms of mercury transport and disposition in the body (see Refs. 1, 5, 6, and 11 and references therein); however, the cellular molecular targets and biochemical processes underlying



**FIGURE 9. Effects of mercurial compounds on Grx in HeLa cells.** *A*, cells were exposed to HgCl<sub>2</sub> or MeHg at different concentrations as indicated for 24 h, and cell lysates were collected for Grx activity determination. Grx activity was expressed as a percentage of untreated control. All data points are means  $\pm$  S.D. of three independent experiments. *B*, recombinant human Grx was fully reduced by DTT, and excess DTT was removed by desalting over a NAP-5 column. HgCl<sub>2</sub> or MeHg was incubated with 10  $\mu$ M of the reduced protein at varying molar ratios as indicated. Remaining free thiol groups were then determined by titration with DTNB. All data points are means  $\pm$  S.D. of two independent experiments. *C*, HgCl<sub>2</sub>-treated and MeHg-treated Grxs appeared as a dimeric and monomeric band, respectively.

mercury toxicity remain to be elucidated. In this study, the objective was to determine whether mercurial compounds would target the Trx and Grx systems. Results of *in vitro* assays showed a rapid loss of mammalian TrxR activity with increasing concentrations of mercury. Inorganic HgCl<sub>2</sub> had greater inhibition potency than organic MeHg, which could possibly be due to the monovalent MeHg being less electrophilic. In addition, as in the case of HgCl<sub>2</sub> and MeHg binding to metallothioneins to form complexes of different coordination geometries (47–50), the different coordination chemistry of HgCl<sub>2</sub> and MeHg toward cysteine residues may also

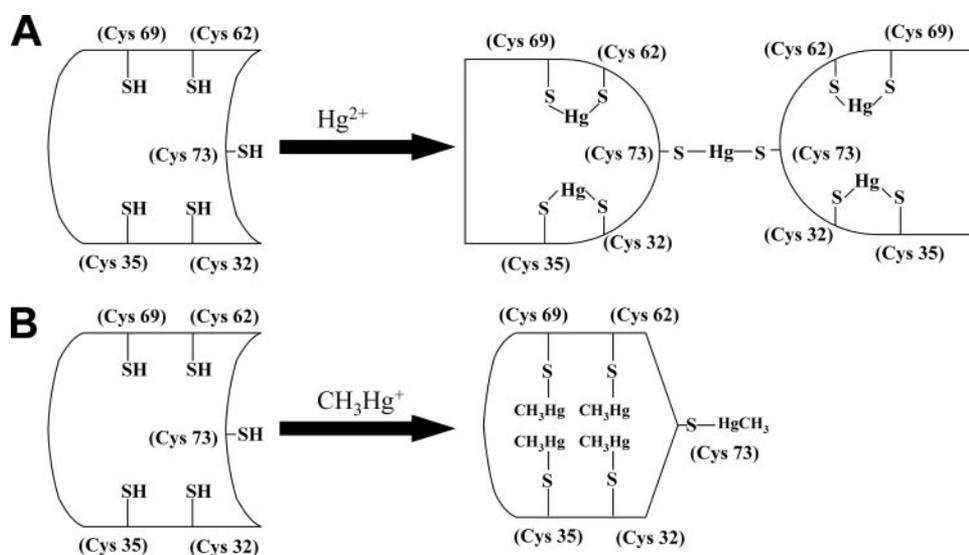


FIGURE 10. Schematic representation of the interaction of mercurial compounds with the five cysteines present in human thioredoxin. *A*,  $\text{HgCl}_2$  caused the formation of dimers, and a molar ratio of 2.5 of inhibitor to Trx was required for the complete loss of free thiols in fully reduced Trx, which is compatible with the existence of 2–3 atoms in the structure, as shown by mass spectrometry analysis. Therefore, it is proposed that two Trx molecules are bridged by  $\text{Hg}^{2+}$  at Cys<sup>73</sup>. Additionally, to the remaining cysteine residues the  $\text{Hg}^{2+}$  ions can form two intramolecular bridges between two cysteine residues (Cys<sup>32</sup>-Cys<sup>35</sup> and Cys<sup>62</sup>-Cys<sup>69</sup>). *B*, based on the results observed in this study, MeHg did not induce Trx dimerization, and the results attained with free thiol titration as well as the mass spectra analysis indicate a ratio of 5 MeHg molecules to 1 of Trx to obtain full oxidation; thus, one MeHg molecule binds to each of the five cysteine residues.

contribute to different interacting capacities with the active site of mammalian TrxR.

Our results showed that both  $\text{HgCl}_2$  and MeHg caused a loss of all five free thiols in fully reduced Trx protein in a dose-dependent mode. This was in agreement with the findings of a previous study, which by means of redox Western blot analysis, has found that metal ions, such as  $\text{Hg}^{2+}$  ( $\text{HgCl}_2$  was used), produce significant oxidation of Trx1 and Trx2 (51).

Mammalian Trx1 has a classic dithiol active site, -Cys<sup>32</sup>-Gly-Pro-Cys<sup>35</sup>-, and three additional structural cysteine residues (Cys<sup>62</sup>, Cys<sup>69</sup>, and Cys<sup>73</sup>). Previous studies have reported how these cysteine residues can participate differently under oxidizing conditions. For instance, a second disulfide motif exists between Cys<sup>62</sup> and Cys<sup>69</sup> that causes a reversible inactivation of Trx (24, 52); intermolecular disulfide formation between Cys<sup>73</sup> leads to dimerization of Trx (32, 53, 54). Our study also revealed that upon treatment of mercurial compounds, hTrx1 dimerization occurred only in the presence of inorganic  $\text{HgCl}_2$ . These results taken together with mass spectrometry analysis indicate the formation of adducts between mercury and the cysteine residues in hTrx1 as well as differential oxidative consequences exerted by different forms of mercury on Trx, as is schematically outlined in Fig. 10. In the case of  $\text{HgCl}_2$ , the mercury ions can 1) bridge two bindings between the active site cysteines and between Cys<sup>62</sup> and Cys<sup>69</sup> and 2) promote dimerization through an intermolecular binding at Cys<sup>73</sup>, which is a highly accessible cysteine, where accumulating evidence shows that it undergoes selective thiol modification (32, 53, 54). Moreover, our results also showed that *E. coli* Trx, which lacks structural cysteines, could not form dimers in the presence of  $\text{HgCl}_2$  and further support the proposed  $\text{HgCl}_2$ -Trx interactions. For MeHg, the interaction with hTrx1 cysteine residues occurs with a 1:1 ratio,

with the hTrx accommodating five MeHg radicals that do not bridge any pair of cysteines, and this consequently may facilitate their removal from hTrx as occurred with DTT treatment.

Several TrxR adducts with mercurial compounds were detected by mass spectrometry analysis<sup>4</sup>; TrxR contains a total of 14 cysteine residues, and the reduced enzyme (59) has three Cys thiols and a Sec selenol in the active sites. The molecular weight changes for oxidized and NADPH-reduced enzyme upon treatment with  $\text{HgCl}_2$  and MeHg show an increased number of interactions involving the active site Sec and Cys residues; however, further analysis in progress will be required to identify all of the molecular mechanisms involved in binding.

Cell-based experiments were carried out to investigate whether the *in vitro* findings with pure enzymes could be extended to intact cells.

Cell viability assays were performed to test the susceptibility of HeLa and HEK 293 cells growth to mercurial compounds; MeHg exhibited higher cytotoxicity than  $\text{HgCl}_2$ . These results are consistent with previous studies, where the concentrations of  $\text{HgCl}_2$  and MeHg that provoke toxic effects on human erythrocytes K-562 cells are 50 and 5  $\mu\text{M}$ , respectively (42), and the  $\text{EC}_{50}$  values of  $\text{HgCl}_2$  and MeHg on undifferentiated pheochromocytoma PC 12 cells are reported to be 5.02 and 0.87  $\mu\text{M}$  respectively (43). Similar observations have been made in bacteria, where the minimal inhibitory concentration of  $\text{HgCl}_2$  and MeHg on *E. coli* K802N (pcFF04) strain is 11.9 and 0.47  $\mu\text{M}$ , respectively (41). Furthermore, MeHg is known to interact with other important cellular systems related to the homeostasis and detoxification mechanisms, such as the GSH system, which may contribute synergistically to MeHg toxicity in cells. A strong inhibition of TrxR activity in HeLa cell lysates within 7 h of exposure to both forms of mercury was observed. In cellular signaling, the Trx system is upstream of a network of pathways, such that inhibition of the Trx system as an early event would trigger a series of downstream signaling pathways and lead to cell death. Mercury-mediated inhibition of the Trx system at 7 h of exposure had indeed led to manifestation of cytotoxic effects between 24 and 48 h. Although to a lesser extent, cellular Trx activity was also inhibited, that could also account for mercury toxicity, since the modification of Trx was recently reported to activate early steps of inflammation and cell adhesion (54). Both TrxR and Trx inhibition in HeLa and HEK 293 cells were correlated to antiproliferative effects exhibited by  $\text{HgCl}_2$  and MeHg, indicating that inhibition of the Trx system

<sup>4</sup> C. M. L. Carvalho, O. Rengby, E. S. J. Arnér, and A. Holmgren, manuscript in preparation.

## Mercury Inhibits the Mammalian Thioredoxin System

accounts at least in part for the cytotoxic effects of mercurial compounds (54). These results were corroborated by a recent work, published while this paper was in preparation, where the authors describe similar effects of HgCl<sub>2</sub> on the Trx system of monocytes (55). Interestingly, between HgCl<sub>2</sub> and MeHg, the less cytotoxic HgCl<sub>2</sub> was more potent in inhibiting purified recombinant mammalian TrxR protein. It could be understood that in cell growth medium solutions, Hg<sup>2+</sup> ions may be chelated or complexed such that the net amount taken up into the cells would be lower than the original dose. Nevertheless, when we compared the effects exerted by concentrations of both compounds used at their respective GI<sub>50</sub> and IC<sub>50</sub> values (Table 2), the extent of inhibition of TrxR activity by HgCl<sub>2</sub> in cell lysates was remarkable. These results, in agreement with the *in vitro* results with purified recombinant mammalian TrxR, strongly suggest that TrxR is targeted by inorganic mercury. The active site of reduced mammalian TrxR contains two adjacent sulfhydryl and selenol groups, which should act as a stronger binding site than isolated thiols or selenols. Therefore, we propose that the enzyme acts as a sink to trap mercury inside the cells. This can be relevant, since it has been reported that after chronic exposure to MeHg, inorganic mercury accounts for more than 80% of mercury within the central nervous system of human and nonhuman primates, indicating that MeHg crosses the blood-brain barrier, and its toxicity develops after demethylation (56, 57). GR and mammalian TrxR share sequence homology and similar three-dimensional structure that includes a FAD- and NADPH-binding domain and an interface region as well as an N-terminal -dithiol/disulfide active site (58–60). In this study, we found that pure GR protein from yeast was susceptible to inhibition by mercurial compounds. However, lysates of HeLa cells treated with HgCl<sub>2</sub> and MeHg for 24 h showed no significant decrease in GR activity, implying that in cells, mercury exhibited selective inhibition on TrxR over GR. Selenium is known to interact with mercury, and it has been suggested as an antagonist to reduce mercury-induced toxicity (11, 16). Selenols (-SeH), having a lower pK<sub>a</sub> (5.3) than thiols (-SH), are fully ionized to selenolates (-Se<sup>-</sup>) under physiological conditions (59, 60) and thus are more reactive toward mercury. It has been reported that MeHg has an inhibitory effect on GPx activity (61, 62), which suggests that the Sec residue in GPx is a potential target. Mammalian TrxR possesses an active site (-Gly-Cys<sup>497</sup>-Sec<sup>498</sup>-Gly) (59) with a penultimate Sec residue, whereas GR lacks the presence of this Sec in its active site. The inhibition of TrxR by mercurial compounds in preference to GR in cells strongly indicates that the C-terminal Sec residue is targeted by mercury. Overall, this specificity offers an explanation of mercury toxic effects at the molecular level as well as highlighting the importance of selenium status in the organism for the prevention of mercury toxicity.

Grx1 is a member of the Trx fold superfamily of proteins (63, 64), which contains a dithiol active site and three structural cysteine residues (Cys<sup>8</sup>, Cys<sup>79</sup>, and Cys<sup>83</sup>). As recently reported, under oxidizing conditions, these structural cysteines are engaged in forming intermolecular, intramolecular, or mixed disulfides with GSH (65). Here, we found that treatment of HgCl<sub>2</sub> or MeHg with fully reduced purified Grx protein caused a loss of free thiols in a dose-dependent manner. Moreover,

consistent with observations made for Trx protein, inorganic HgCl<sub>2</sub>, but not MeHg resulted in Grx dimerization. Notably, mercurial compounds at doses that provoked marked inhibition of TrxR and Trx activity in cells did not produce any significant effect on Grx activity, which again indicates the specific inhibitory effect of mercury on the Trx system.

In the research field of mercury toxicology, it has been reported that reactive oxygen species production mediates mercury cytotoxicity (66, 67) and that mercury interacts with the GSH system, causing its depletion (12, 61, 62). The thioredoxin system is an electron donor for peroxiredoxins, which eliminate peroxides, and inhibition by mercury would increase reactive oxygen species. Our study serves to elucidate whether inhibition of the two thiol redox systems Trx and Grx is involved in mercury-mediated cytotoxicity. We have demonstrated that in a cellular system, the inorganic and organic forms of mercury preferentially target the Trx system (but not GR and Grx), of which TrxR was inhibited to a greater extent than Trx by more potent mercury concentrations. Biomarkers are tools used in human studies to assess exposure and disease risks. By far, only hair and blood mercury concentrations are considered valid and specific biomarkers for human exposure to MeHg (1, 7, 28). Therefore, predictive biomarkers, especially those of early effect, such as changes in protein (enzymatic activity and/or level of expression) would be of utmost value to predict and prevent the development of serious toxicity manifested in the human population. The reduction in activity detected on the cellular Trx system, especially that of TrxR, indicates its potential for being tested as a biomarker of mercury toxicity.

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