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Research Paper

Impaired cross-talk between the thioredoxin and glutathione systems is related to ASK-1 mediated apoptosis in neuronal cells exposed to mercury

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ABSTRACT

Mercury (Hg) compounds target both cysteine (Cys) and selenocysteine (Sec) residues in peptides and proteins. Thus, the components of the two major cellular antioxidant systems – glutathione (GSH) and thioredoxin (Trx) systems – are likely targets for mercurials. Hg exposure results in GSH depletion and Trx and thioredoxin reductase (TrxR) are prime targets for mercury. These systems have a wide-range of common functions and interaction between their components has been reported. However, toxic effects over both systems are normally treated as isolated events.

To study how the interaction between the glutathione and thioredoxin systems is affected by Hg, human neuroblastoma (SH-SY5Y) cells were exposed to 1 and 5 μM of inorganic mercury (Hg^{2+}), methylmercury (MeHg) or ethylmercury (EtHg) and examined for TrxR, GSH and Grx levels and activities, as well as for Trx redox state. Phosphorylation of apoptosis signalling kinase 1 (ASK1), caspase-3 activity and the number of apoptotic cells were evaluated to investigate the induction of Trx-mediated apoptotic cell death. Additionally, primary cerebellar neurons from mice depleted of mitochondrial Grx2 (mGrx2D) were used to examine the link between Grx activity and Trx function.

Results showed that Trx was affected at higher exposure levels than TrxR, especially for EtHg. GSH levels were only significantly affected by exposure to a high concentration of EtHg. Depletion of GSH with buthionine sulfoximine (BSO) severely increased Trx oxidation by Hg. Notably, EtHg-induced oxidation of Trx was significantly enhanced in primary neurons of mGrx2D mice. Our results suggest that GSH/Grx acts as backups for TrxR in neuronal cells to maintain Trx turnover during Hg exposure, thus linking different mechanisms of molecular and cellular toxicity. Finally, Trx oxidation by Hg compounds was associated to apoptotic hallmarks, including increased ASK-1 phosphorylation, caspase-3 activation and increased number of apoptotic cells.

1. Introduction

Exposure to mercury (Hg) compounds causes neurotoxicity that can range from subtle changes in cognitive and motor development to severe impairment of neuromotor functions, depending on the length and magnitude of exposure [1]. Inhibition of glutamate transport, hindrance of neuronal migration and oxidative stress are known hallmarks of mercury mediated neurotoxicity [2]. On the molecular level, Hg has a high affinity to bind thiols and selenols [3–5] and consequently both the thioredoxin and glutathione systems are targets for mercurials.

The thioredoxin system comprises thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, and is a major protein disulphide reductase system [6]. The diverse cellular functions relying on the activity of this system include DNA synthesis, ROS scavenging, regulation of cell signalling and protein folding and repair [7]. Oxidation of Trx results in decreased antioxidant capacity and dysregulation of specific proteins, namely peroxiredoxins (Prx) [8], and activates ASK-1, a MAPKKK that regulates the p38 apoptotic pathway and subsequent downstream molecular signalling, which leads to caspase mediated cell death [9].

The selenoenzyme TrxR is extremely prone to inhibition by mercurials both in vitro [3,4,10] and in vivo [11,12]. For example, in HeLa cells exposed for 24 h to MeHg the IC_{50} for TrxR was 1.4 μM whereas for the homologous enzyme, glutathione reductase (GR), it was > 20 μM [3]. This sensitivity is due to the reactivity and position of the selenocysteine (Sec) residue in the open C-terminal of TrxR's active site

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[13]. Mercury compounds also target thioredoxin (Trx), binding Cys32 and Cys35 at the active site as well as structural Cys at positions 62, 69, and 73, albeit higher doses are required to achieve full inhibition [3,11]. However, the mechanism leading from inhibition of the Trx system by Hg compounds to cell death is not clarified.

Glutathione is the most abundant cellular thiol [14], and is involved in the detoxification of electrophilic xenobiotics such as Hg compounds [15–17]. In addition, GSH acts as an electron donor for glutathione peroxidases (GPx) and the redox active Cys residues in glutaredoxins (Grx). Although depletion of GSH by Hg compounds has been largely reported [16–20], we recently demonstrated an increase of GR activity following methylmercury (MeHg) exposure, which is a possible compensatory mechanism to increase available GSH and restore cellular antioxidant capacity [11].

The thioredoxin and glutathione systems have some overlapping functions allowing maintenance of redox regulation, even if one of the systems is negatively affected [21]. One important example is that both Trx and Grx are efficient electron donors for RNR [22]. In addition to redundancy in function, a molecular cross-talk between both systems occurs to assure normal function when one of the components is hampered [23–26]. However, toxicity studies overlook this fact and usually report isolated effects of mercury over these antioxidant systems.

Therefore, the aim of this study was to dissect the interaction between the glutathione and thioredoxin systems in neuronal cells during exposure to mercury compounds establishing its relevance for the development of Hg toxicity. Moreover, we considered how inhibition of the Trx system by mercurials regulates cell death.

2. Materials and methods

2.1. Cell culture

Human neuroblastoma cells (SH-SY5Y) were purchased from ATCC® and cultured in Eagle's Minimum Essential Medium (ATCC®) supplemented with 10% fetal bovine serum (Gibco®) and 1% Pen-Strep mixture (Gibco®) in a humidified incubator at 37 °C and 5% CO_2.

2.2. LDH assay

General cell death was assessed by, evaluating lactate dehydrogenase (LDH) release from cells using the Cytotoxicity Detection KIT^{Plus} (LDH) from Roche. Briefly, SH-SY5Y cells were seeded in 96 well plates (5×10^3 cells/well) and grown for 24 h, until mercury compounds ($HgCl_2$; CH_3HgCl; C_2H_5HgCl from Sigma, hereafter referred as Hg^{2+}, MeHg and EtHg, respectively) were added in different concentrations (0, 1, 5, 10, 25, 50 μM). After 24, 48 and 72 h of exposure, the supernatant was collected and LDH Assay Lysis solution was added. Both the supernatant and the lysate were assessed for LDH activity after adding lactate (substrate), tetrazolium salt, and NAD^+ (cofactor), according to manufacturer's instructions. Plates were protected from light and incubated for 30 min at RT. Absorbance was measured at 490 nm, with 620 nm as reference, in a microplate reader (Zenyth3100, Anthos Labtec Instruments), and LDH release was quantified as the ratio between the amount in the supernatant and total LDH (supernatant + lysate). The LC_{50} for each mercury compound was calculated as the concentration causing a 50% increase in LDH release from cells.

2.3. Preparation of cell lysates

SH-SY5Y cells (1×10^6) were plated in 10 mm Petri Dishes and grown until 80% confluence. At the start of each experiment, fresh medium was supplied to cells followed by the addition of Hg^{2+}, EtHg or MeHg (1 and 5 μM). Exposure lasted 6 or 24 h after which cells were harvested, washed in PBS, centrifuged and the pellet resuspended in the appropriate lysis buffer, depending on the requirements of each specific protocol. For measuring enzymatic activities and analysing protein expression by Western blot, cells were disrupted in cell lysis buffer containing 25 mM Tris–Cl (pH7.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 20 mM NaF, 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate; 20 mM sodium β-glycerophosphate, 0.5% TritonX-100, and 1 tablet of protease inhibitor cocktail (Roche) per 10 ml. After incubation on ice for 30 min with vortexing every 5 min, samples were frozen at −20 °C until analysis. Before activity assays, samples were centrifuged at 13,000*g* for 10 min at 4 °C and the pellet discarded. These lysates were used to quantify Grx activity and expression as described below.

2.4. Cell fractionation

Sub-cellular fractions of SH-SY5Y cells were obtained following the method described in Branco et al. [27]. Briefly, after exposure to 1 μM of Hg^{2+}, EtHg or MeHg for 24 h, cells were washed and suspended in mitochondrial isolation buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes–NaOH, pH7.5) with protease inhibitor cocktail (1 tablet/10 ml; Roche). Cells were lysed using a Teflon pestle (40–50 strokes) followed by centrifugation at 600*g* for 10 min at 4 °C. Nuclear pellets and cellular debris were discarded and supernatants centrifuged at 13,000*g* for 15 min at 4 °C to obtain mitochondrial pellets and supernatant soluble fractions. Mitochondrial pellets were treated with lysis buffer and the supernatant was centrifuged for 1 h at 100,000*g* and 4 °C to obtain the cytosolic fraction. These fractions were used to quantify TrxR activity and expression as described below.

2.5. Total protein determination

Total protein in all samples was quantified by mixing each sample with diluted (5x) Coomassie dye (Bio-Rad) in 96-well plates, with subsequent measurement of absorbance at 595 nm in a microplate reader [28]. Protein concentration was calculated from a calibration curve using BSA as a standard.

2.6. Thioredoxin reductase activity measurement

Thioredoxin reductase activity was measured in sub-cellular fractions following the protocol described by Arnér and Holmgren [29]. Briefly, 25 μg of the mitochondrial or cytosolic fractions of SH-SY5Y cells were incubated in 96-well plates with 0.3 mM insulin, 660 μM NADPH, 3 mM EDTA, and 5 μM human Trx1 (IMCO Corp., Sweden), in 85 mM Hepes buffer (pH 7.6) at 37 °C for 20 min. Human Trx1 was previously reduced with dithiothreitol (DTT) at 37 °C and desalted in a NAP-5 column. Control wells containing the same reagents but excluding Trx addition were prepared in parallel. After the incubation period, the reaction was stopped by addition of 250 μL of a 1 mM DTNB solution in guanidine-HCl (6 M, pH 8.0). Absorbance was measured in a microplate reader (Zenyth3100, Anthos Labtec Instruments) at 412 nm, and TrxR activity quantified as the difference in absorbance between the Trx and controls, using a recombinant TrxR1 standard curve.

2.7. Glutaredoxin activity

Glutaredoxin activity was measured in whole cell lysates by the HED assay, as described by Holmgren and Aslund [30] with some modifications. Briefly, 5 μL of cell lysate were incubated with 1 mM GSH, 200 μM NADPH, 6 μg/ml GR and 0.7 mM of 2-hydroxyethyl disulphide (HED) in TE/BSA buffer (50 mM Tris, 2 mM EDTA, 0.1 mg/ml BSA), pH 8.0. Activity was evaluated following the consumption of NADPH at 340 nm for 5 min, and normalized for protein concentration. A standard curve using hGrx1 was used to quantify activity in samples.

2.8. Total glutathione and glutathione disulphide

For measuring the effect of exposure to mercurials over GSH, cells were lysed with a Teflon pestle in ice-cold extraction buffer (0.6% sulfosalicylic acid and 0.1% Triton-X in 0.1 M potassium phosphate buffer with 5 mM EDTA, pH 7.5) and sonicated for 2 min in ice-cold water. The cell lysate was then centrifuged (3000*g* for 5 min, at 4 °C) and the supernatant was used to measure total GSH and GSSG content [31]. For assessing total GSH, 5 µL of each sample were incubated with 5 mM DTNB, 1 mM NADPH and 50 nM GR in phosphate buffer + EDTA, and TNB formation was monitored at 412 nm for 2 min [31]. For measuring GSSG, the same procedure was applied after the supernatants were derivatized for 1 h at RT with 2-vinylpyridine (Sigma) and neutralized with triethanolamine [31]. GSH and GSSG were quantified from calibrations curves using either GSH or GSSG as standards and normalized for total protein content.

2.9. Expression levels of TrxR and Grx

Western blot was used to determine the effect of mercury exposure in the expression levels of TrxR1/TrxR2 (sub-cellular fractions) and Grx1/Grx2 (whole-cell lysates). Samples (40 µg of protein) were separated by SDS–PAGE on a 4–12% Bis–Tris gel with MES running buffer (Invitrogen) under reducing conditions, transferred to a nitrocellulose membrane, blocked with a 5% skimmed milk solution and probed with the appropriate primary and secondary antibodies. The following antibodies were used: anti-human TrxR1 rabbit polyclonal IgG (sc-20147, Sta. Cruz), anti-human TrxR2 mouse monoclonal IgG (sc-365714, Sta. Cruz), anti-human Grx1 goat polyclonal IgG (AGRX-03, IMCO Corp.), anti-human Grx2 rabbit polyclonal IgG (AGRX-02, IMCO Corp.), anti-human GAPDH rabbit polyclonal IgG (sc-25118, Sta. Cruz), anti-human VDAC rabbit polyclonal IgG, goat anti-rabbit IgG–HRP (sc-2004, Sta. Cruz), rabbit anti-goat IgG–HRP (sc-2768; Sta. Cruz) and goat anti-mouse IgG–HRP (sc-2005; from Sta. Cruz). Given the overlapping molecular weight between TrxR1 and TrxR2 and between Grx1 and Grx2, membranes were first probed for one of the enzymes (e.g. TrxR2) and then treated with H_2O_2 before reprobing for the other isoform (e.g. TrxR1), according to the method described by Sennepin et al. [32].

Expression levels were normalized for protein loading on the gel, which was assessed by either evaluating housekeeping proteins (GAPDH, VDAC) or Ponceau S staining prior to the blocking step. All Western blots were performed in at least three independent experiments.

2.10. Trx oxidation state (redox western blot)

The redox state of Trx1 and Trx2 was assessed by evaluating the number of reduced SH groups (Free SH), according to the Protein Electrophoretic Mobility Shift Assay established by Bersani et al. [33] and modified by Du et al. [23]. After exposure to mercury compounds and collection as described above, cell pellets were resuspended in Sample Solution (SS, Tris 50 mM, Urea 8 M, pH 8.3) containing 30 mM of iodoacetic acid (IAA). After mixing, samples were placed for 30 min at 37 °C, followed by centrifugation at 16,000*g* for 10 min. The resulting supernatant was washed in acetone: HCl mix (98:2), centrifuged for 10 min at 16,000*g*, and the precipitate was resuspended in SS containing 5 mM of DTT. After 30 min at 37 °C, iodoacetamide (IAM; 10 mM) was added and samples incubated at 37 °C for 30 min.

After alkylation was completed, samples (100 µg of protein) were loaded in a Urea-PAGE gel (Stacking Gel: 2.5% acrylamide; Running Gel: 12% acrylamide; 8 M Urea) and run at 10 mA for 2 h. Afterward, the proteins in the gel were transferred to a nitrocellulose membrane, blocked with skimmed milk and incubated with primary antibodies for human Trx1 (ATRX8, IMCO Corp.) and Trx2 (sc-50335, Sta. Cruz) and appropriate secondaries, as previously described. Mobility standards corresponding to the different oxidation sates of Trx1 and Trx2 were prepared in parallel by reducing enzymes in cells with SS containing 5 mM DTT followed by incubation at 37 °C with different ratios of IAA and IAM. These standards were loaded in the Urea-PAGE along with the samples. Since human Trx1, has 5 Cys residues (2 at the active site plus 3 structural), 6 possible oxidation states exist from fully reduced (5 free SH groups) to fully oxidized (0 free SH groups), whereas only three possibilities exist for Trx2, since it only has the 2 Cys at the active site: fully reduced (2 free SH groups), fully oxidized (0 free SH groups), and the intermediate state (1 free SH).

To test the effect of GSH depletion over the oxidation of Trx1 and Trx2 caused by exposure to mercurials, SH-SY5Y cells were treated for 16 h with 100 µM of DL-buthionine-[S,R]-sulfoximine (BSO) followed by addition of Hg compounds and evaluation of enzyme oxidation as described above.

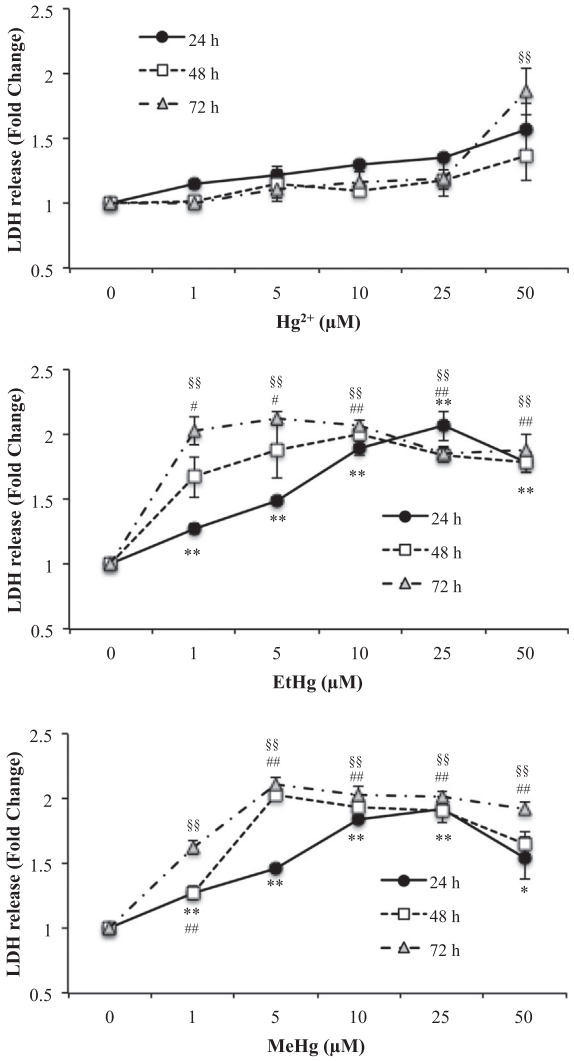

Fig. 1. General cell death as assessed by LDH assay after exposure of SH-SY5Y cells to mercury compounds. Cells were exposed for 24, 48 and 72 h to different concentrations of Hg^{2+}, EtHg or MeHg. LDH release into the culture media was quantified and the LC_{50} calculated. Data are expressed as mean ± (SEM) of at least 3 independent experiments. The *, # and § symbols refer to the statistical analysis for the 24, 48 and 72 h time-point, respectively. *, #, § $p < 0.05$ from control; **, ##, §§ $p < 0.01$ from control.

2.11. Peroxiredoxin 3 oxidation state

Cells treated with Hg compounds were probed for the oxidation state of peroxiredoxin 3 (Prx3) following the method described by Brown et al. [34]. After collection, cells were resuspended for 15 min in NEM lysis buffer consisting of 50 mM NaCl, 1 mM EDTA, 1 mM EGTA,

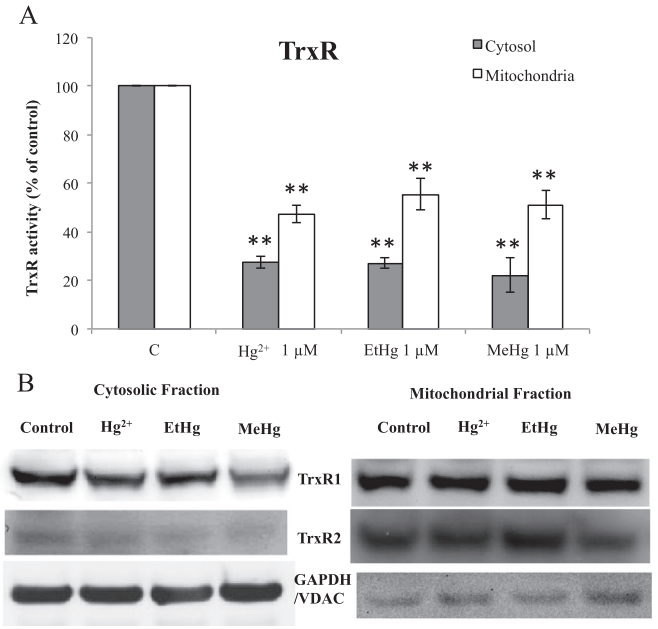

Fig. 2. Inhibition of TrxR by mercury compounds in SH-SY5Y cells. Cells were exposed for 24 h to 1 µM of Hg^{2+}, EtHg or MeHg and fractioned to separate the mitochondrial and cytosolic fractions. TrxR activity was measured by the insulin reduction assay (A) and TrxR1 and TrxR2 expression was evaluated by Western blot (B) in each sub-cellular fraction. Data are representative of 3 independent experiments. ** $p < 0.01$ from control.

100 mM N-ethylmaleimide (NEM) and protease inhibitor cocktail in 40 mM HEPES, pH 7.4. Afterward, 1% CHAPS was added to samples to lyse cells and supernatants were collected after centrifugation for 4 min at 15,000*g*. Forty µg of the soluble protein fraction were separated by SDS-PAGE electrophoresis on a 4–12% Bis-Tris Mini gel in non-reducing conditions, transferred to a nitrocellulose membrane, blocked in 5% skimmed milk and probed with anti-Prx 3 rabbit polyclonal antibody (LF-PA0030, Ab Frontier) and goat anti rabbit secondary antibody. The ratio between the bands of Prx3 dimer and monomer was quantified for each sample using the Bio-Rad Quantity One software.

2.12. Protein S-glutathionylation

S-glutathionylated proteins (PSSG) were measured by Western blot using a mouse monoclonal anti-GSH antibody (101-A-100, Virogen). To optimize PSSG detection, cells were disrupted in lysis buffer (25 mM Tris–Cl, pH7.5; 100 mM NaCl; 2.5 mM EDTA; 2.5 mM EGTA; 20 mM NaF; 1 mM sodium orthovanadate; 20 mM sodium pyrophosphate; 20 mM sodium β-glycerophosphate; 0.5% TritonX-100; protease inhibitor cocktail) supplemented with 25 mM of NEM. After centrifugation (13,000*g* for 10 min, 4 °C) to obtain the soluble fraction, samples (70 µg protein) were separated by SDS-PAGE in non-reducing conditions, transferred onto a nitrocellulose membrane and blocked for 2 h with a 5% BSA solution containing 2.5 mM NEM prior to probing with the GSH antibody [35]. Levels of PSSG were normalized for total protein loading, which was evaluated by Ponceau S staining of the nitrocellulose membrane prior to the blocking step.

2.13. ASK-1 activation

ASK-1 activation was assessed in whole cell lysates (lysed as described previously) by looking at the phosphorylation of Thr838 by western blot. Samples were run in SDS-page as described above and probed with anti pASK-1 (Thr845) antibody from rabbit (sc-109911; Sta. Cruz) followed by the appropriate secondary antibody. p-ASK-1 was normalized for total ASK-1 levels, which were assessed by probing with a rabbit polyclonal anti-ASK-1 antibody (sc-7931; Sta. Cruz).

2.14. Caspase activity

General caspase-3-like activity was evaluated by measuring the enzymatic cleavage of chromophore p-nitroanilide (pNA) from the substrate N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA). Briefly, samples (100 µg of protein) were mixed with 50 mM of DEVD-pNA in the presence of 2 mM DTT and incubated for 3 h at 37 °C, after which absorbance was measured at 405 nm in a microplate reader.

2.15. Guava ViaCount assay

To evaluate viable, apoptotic, and dead cell populations in SH-SY5Ycells exposed to 5 µM of each Hg compound (Hg^{2+}, EtHg, MeHg) and vehicle controls, the viaCount assay was used with the Guava easyCyte 5HT flow cytometer (GuavaTechnologies, Inc., Hayward, CA, USA). The ViaCount Assay distinguishes viable and non-viable cells based on differential permeability of two dyes in the Guava ViaCount Reagent. The membrane-permeant dye stains all nucleated cells, leaving the cellular debris unstained, while the membrane-impermeant dye brightly stains damaged cells, thus indicating apoptotic and dying cells. After 24, 48 and 72 h of treatment, cell culture supernatants were collected and adherent cells were detached with TrypLE (Invitrogen). Next, detached cells were pooled with cell culture supernatants and centrifuged for 5 min (650*g*). Supernatants were discarded and the cells were resuspended in phosphate buffered saline (PBS). Subsequently, 15 µL of cell suspension were mixed with 135 µL of Guava ViaCount reagent, and incubated for 5 min at room temperature. Sample acquisition and data analysis were performed using the ViaCount software module.

2.16. Mitochondrial glutaredoxin 2 deletion mice

The mouse model with a deletion of the mitochondrial localization signal of Glutaredoxin 2 (hereafter abbreviated mGrx2D, mitochondrial-Grx2-Deletion) was generated by targeting exons 1c, 1a and 2 and replacing them by a Neomycin cassette, which was subsequently excised (Supplementary material Fig. S1) (Central Institute for Experimental Animals, Japan; http://www.ciea.or.jp). This deletion caused a significant decrease in Grx2 expression and Grx activity in the mitochondria (Supplementary material Fig. S2). Animal housing, handling and experimentation were approved by the Regional Animal Ethics Committee of Northern Stockholm.

2.17. Mouse cerebellar granular neurons primary culture

Isolation of primary culture of cerebellar granule neuron from wild-type (wt) and mGrx2D postnatal pups, was performed as previously described by Lee and co-workers [36]. Briefly, 5-day old wt and mGrx2D mice pups were euthanized and immediately the brains were collected and placed in ice-cold Hanks' Balanced Salt Solution (HBSS, ThermoFisher Scientific) with 30 µg/ml BSA and 1.2 mM $MgCl_2$. Afterwards, the meninges were removed and the cerebella were dissected. The cerebella were transferred for digestion into the same buffer supplemented with 1% trypsin (GIBCO) and 0.5 µg/ml DNase (Roche). After 15 min of incubation at 37 °C, an equal volume of HBSS, supplemented with 20% FBS (Fisher Scientific) and 0.6 µg/ml DNase was added to stop the trypsin digestion. Samples were centrifuged at 800*g* for 5 min and the pellets were resuspended and gently triturated in the HBSS-20% FBS-DNase buffer solution, and centrifuged again. The final cell pellets were resuspended in complete Neurobasal culture medium (Life Technologies) supplemented with 2% B27 (Life Technologies), 0.5 mM GlutaMax (Life Technologies), 20 mM KCl and Penicillin-Streptomycin (0.01 U/ml–0.01 µg/ml), and cells were plated into Petri dishes coated with poly-L-lysine (Sigma-Aldrich). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C and used for experiments after 5 days. Differentiated cells were treated with 2.5 or

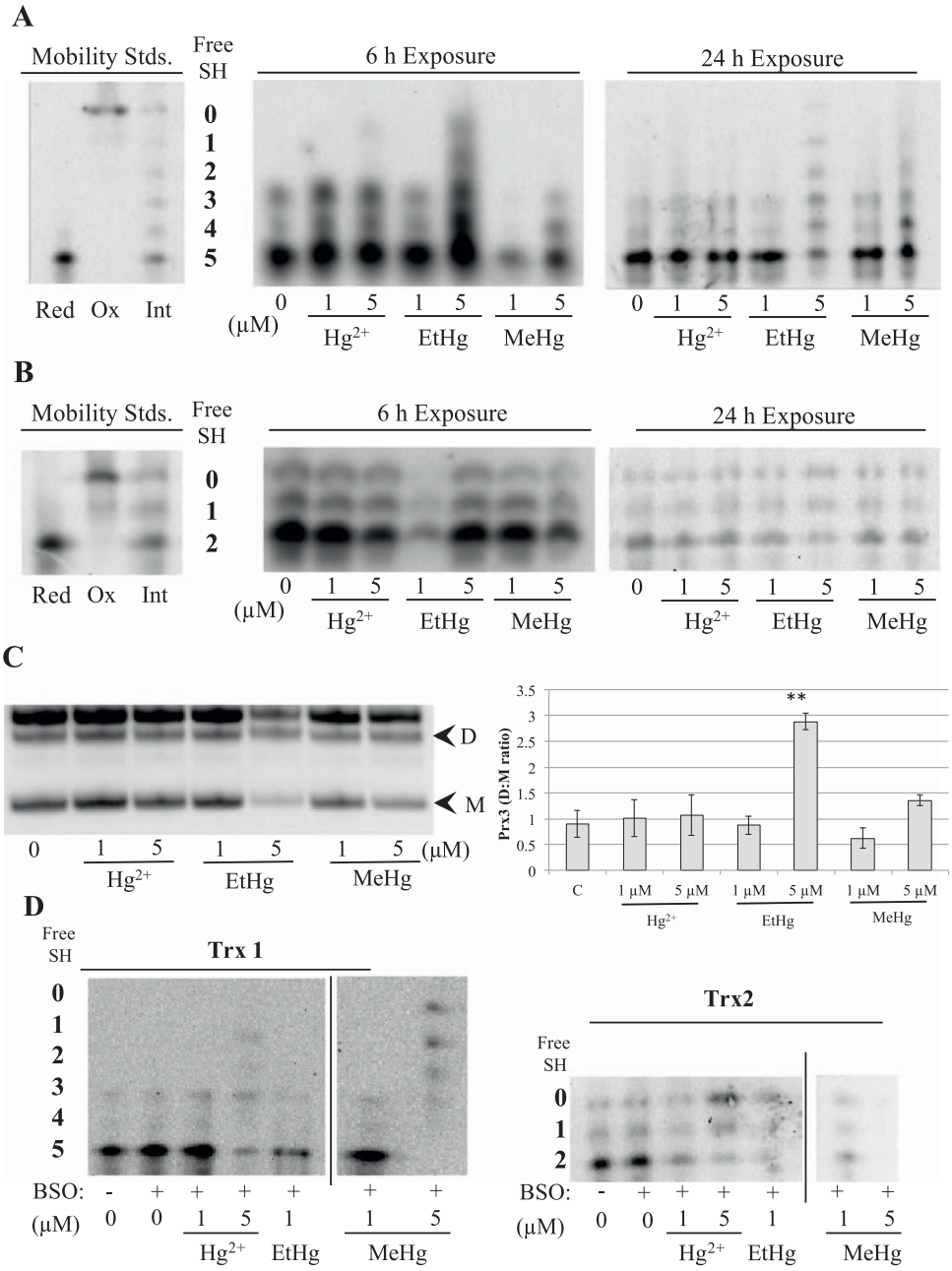

Fig. 3. Effect of exposure to mercury compounds on the oxidation state of thioredoxin 1, thioredoxin 2 and peroxiredoxin 3 in SH-SY5Y cells. Cells were exposed for 6 or 24 h to 1 and 5 μM of Hg^{2+}, EtHg or MeHg and the redox state (number of free SH groups) of Trx1 (A), Trx2 (B) and Prx3 (C) (24 h only) was analyzed by Western blot. Mobility standards corresponding to the different oxidation states of either Trx1 (6 possible states because of 5 Cys residues) or Trx2 (3 possible states because of 2 Cys residues) were run alongside samples. The consequence of pre-treatment (16 h) with BSO over the effect of Hg compounds (24 h) and Trx1 and Trx2 redox state was also analyzed (D). Blots are representative of at least 3 independent experiments. ** $p < 0.01$ from control. Red – fully reduced state; Ox – fully oxidized state; Int – intermediate oxidation states; D – dimer; M – Monomer. The vertical line represents the site where the membrane was cut, corresponding to exposure to EtHg (5 μM) after pre-treatment with BSO, which resulted in extended cell death and low protein levels that could not be detected by Western blot.

5 μM of EtHg for 24 h. Samples were washed with cold PBS and all the lysates were processed according to the protocol previously described for the redox Western blot.

2.18. Statistical analysis

Results in figures are presented as mean ± standard error (S.E.) of at least 3 independent experiments. Differences between groups were determined with a *t*-test for independent samples and considered significant at $p < 0.05$ and very significant at $p < 0.01$.

3. Results

3.1. Cytotoxicity of Hg compounds in SH-SY5Y cells

The citotoxicity of Hg compounds was assessed by evaluating LDH release from SH-SY5Y cells after exposure to Hg^{2+}, EtHg or MeHg. Results showed that the toxicity of EtHg > MeHg > > > Hg^{2+} (Fig. 1), with the respective LC_{50} after 24 h of exposure being 4.6, 4.9 and 40.8 μM. These results are in agreement with the general notion that organomercurials are more cytotoxic than inorganic forms and that EtHg has a slightly higher toxicity than MeHg [10].

3.2. Effect of Hg exposure on the Trx system in SH-SY5Y cells

Thioredoxin reductase activity was significantly affected by exposure to 1 μM of all mercury compounds, both in cytosolic (> 70% inhibition; $p < 0.01$) and mitochondrial fractions (> 40% inhibition; $p < 0.05$) of SH-SY5Y cells (Fig. 2A). Expression of both TrxR1 and TrxR2 was slightly decreased, especially by exposure to organomercurials (Fig. 2B).

For the specific concentrations (1 and 5 μM) and time-points (6 and 24 h) analyzed, Hg^{2+} failed to cause any significant change in Trx1 and Trx2 oxidation states (Fig. 3A and B). Also, no significant increase in the oxidation of both Trx isoforms could be noted at 1 μM of either EtHg or MeHg (Fig. 3A and B). In fact, oxidation of Trx1 only became evident

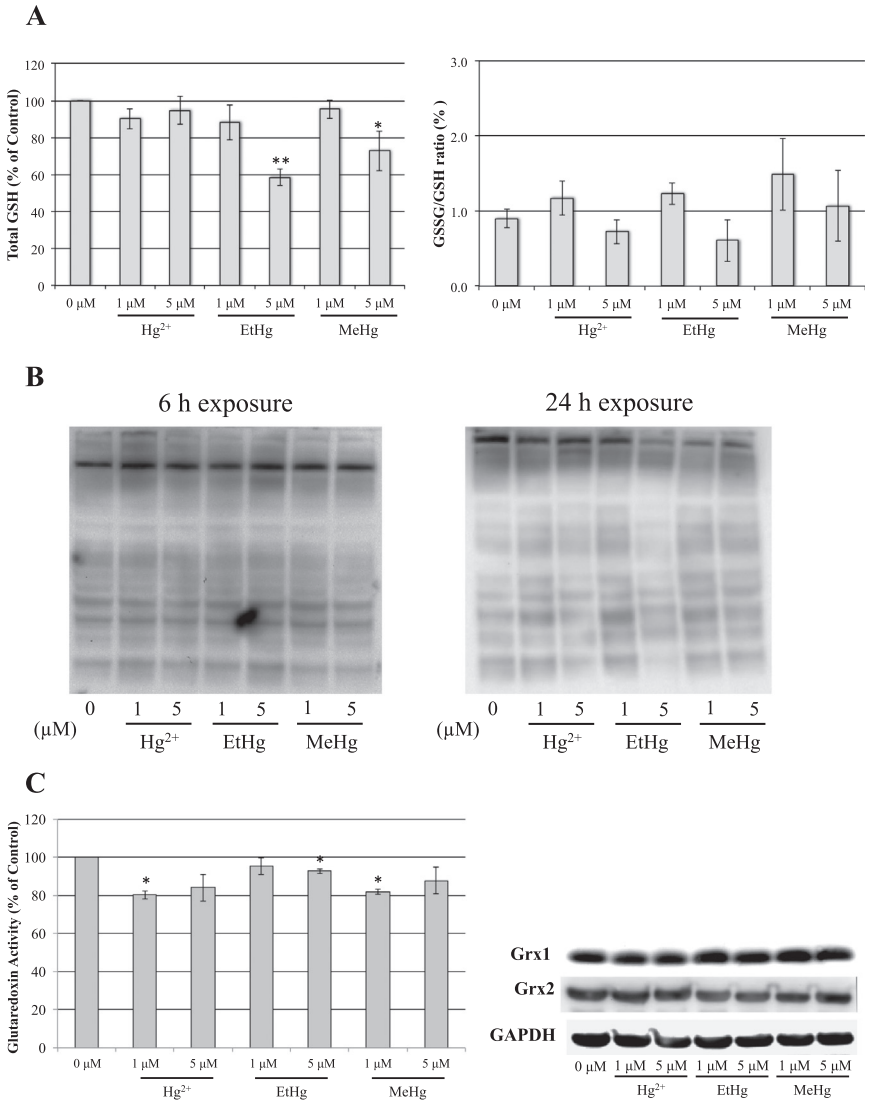

Fig. 4. Effect of exposure to mercury compounds on the glutathione/glutaredoxin system in SH-SY5Y cells. Cells were exposed to 1 and 5 µM of Hg^{2+}, EtHg or MeHg for 24 h and the effect over total GSH (A) and GSSG/GSHtot (B) was analyzed. Additionally, the effect of mercurial on PSSG was analyzed by Western blot (6 and 24 h exposure) (C). Glutaredoxin activity was quantified by the HED assay and Grx1 and Grx2 expression was evaluated by Western blot following a 24 h exposure to the different mercury compounds (D). First the membrane was incubated with Grx1 antibody and respective secondary. After development with chemiluminescent substrate the membrane was treated with H_2O_2 and incubated with the appropriate antibodies for Grx2 detection (see Section 2 for details). Data are representative of at least 3 independent experiments. ** $p < 0.01$ and * $p < 0.05$ from control.

when the concentration of these compounds increased to 5 µM. Regarding EtHg, Trx1 oxidation was already noted after 6 h of exposure (Fig. 3A). Trx2 was less affected than its cytosolic counterpart with oxidation increasing following a 24 h exposure to 5 µM EtHg (Fig. 3B). This rise in oxidation of Trx2 was accompanied by a 3-fold increase in Prx3 dimerization (Fig. 3C).

Pre-treatment of SH-SY5Y cells with BSO resulted in a 75% decrease in the levels of GSH (data not shown). Following exposure to mercurials, pre-treated cells presented much higher oxidation of both Trx1 and Trx2 (Fig. 3D). In fact, following GSH depletion, 5 µM Hg^{2+} increased the oxidation of mitochondrial and cytosolic Trx. Exposure to 5 µM of MeHg produced many dead cells and thus protein load in the gel was low with only faint bands at the most oxidized sates being observed. Cell death was even more significant under exposure to 5 µM EtHg and thus, Trx's oxidation state could not be evaluated (Fig. 3D).

3.3. Glutathione levels and S-glutathionylation

Total GSH levels were significantly decreased by exposure to 5 µM of EtHg ($p < 0.01$) and MeHg ($p < 0.05$), with 40% and 20% reduction in available GSH, respectively (Fig. 4A). The ratio between GSSG and GSH was kept low (≈ 1%) in all treatments (Fig. 4B). Protein S-glutathionylation was unchanged after 6 h of exposure to mercurials but as treatment extended to 24 h, a decrease in the amount of glutathionylated proteins was evident in cells exposed to 5 µM of EtHg.

3.4. Glutaredoxin activity and consequence of mitochondrial Grx2 deletion

In SH-SY5Y cells, activity of Grx was only slightly affected by exposure to Hg compounds (< 20%; $p < 0.05$). No change in the expression of either Grx1 or Grx2 was noted (Fig. 4C).

To evaluate a possible role of Grx in protecting Trx from oxidation, primary cultures of cerebellar granular neurons from mGrx2D mice were exposed to 2.5 and 5 µM of EtHg, which resulted in a significant dose-dependent oxidation of Trx2 as seen by redox Western Blot (Fig. 5). On the other hand, Trx2 in primary neurons from wild-type mice was more resistant to oxidation by EtHg with no significant increase in oxidation following exposure to 5 µM (Fig. 5).

3.5. ASK-1 phosphorylation, caspase activation and apoptosis

Phosphorylation of ASK-1 was analyzed by Western blot (Fig. 6A). Increased levels (2-fold) of pASK-1 were found in SH-SY5Y cells exposed to 5 µM of EtHg for 24 h, which is consistent with the increased oxidation levels of Trx1 and Trx2 previously observed. Furthermore, caspase-3 activity was also significantly enhanced in this condition (Fig. 6B).

Methylmercury (5 µM) also led to higher pASK-1 levels but not as markedly as EtHg, and no increase in caspase-3 was observed (Fig. 6A and B). These results are consistent with the ViaCount Assay which showed an earlier increase (24 h of exposure) in the number of

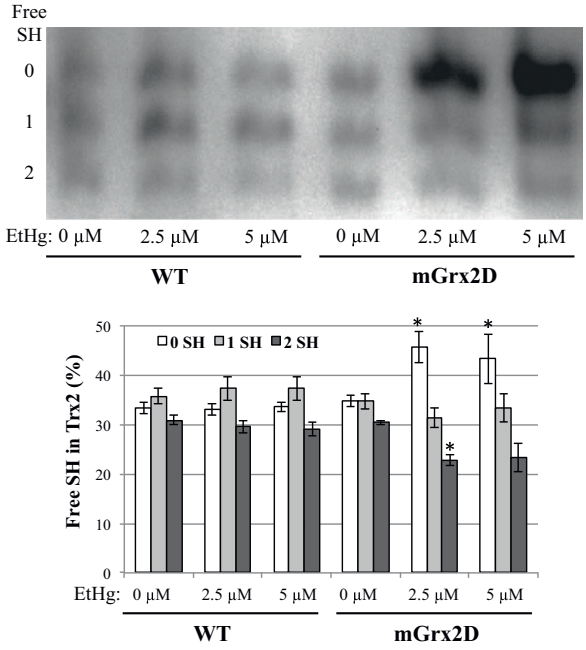

Fig. 5. Oxidation of Trx2 in cerebellar neurons of mice with mitochondrial glutaredoxin 2 depletion (mGrx2D). Primary cultures of cerebellar granular neurons from wild-type (WT) and mice depleted of Grx2 in mitochondria (mGrx2D) were exposed for 24 h to 2.5 and 5 μM of EtHg, after which the oxidation state of Trx2 was analyzed by redox Western blot. Data are representative of 3 independent experiments. * $p < 0.05$ from control.

apoptotic cells upon exposure to EtHg, compared to MeHg where the increase in the number of apoptotic cells was only significant following 48 h of exposure (Fig. 6B).

4. Discussion

The present study contributes to a better understanding of the involvement of the thioredoxin and glutathione systems in the toxicity of mercury compounds and discloses the relation between Trx oxidation by Hg and the development of cell death.

Thioredoxin reductase is an early target of Hg with its inhibition arising at levels (1 μM) markedly below the LC_{50} for all Hg compounds. This is in agreement with previous data from our group [3,4], and can be attributed to the high reactivity of the Sec residue in the open C-terminal of TrxR's active site [4,13]. Most importantly, it is clear that impairment of TrxR activity can be considered as an early effect biomarker of Hg toxicity [37]. In SH-SY5Y cells, cytosolic activity of TrxR is more affected than mitochondrial activity, while no up-regulation of TrxR1 expression is observed. This contrasts with previous observations in HepG2 cells [27], where Nrf-2 mediated signalling prevented the loss of TrxR1 activity by promoting transcription and increasing de novo synthesis.

As we had previously reported [3,11], despite TrxR1 breakdown, Trx1 was kept in a reduced state in SH-SY5Y cells exposed to Hg compounds (Fig. 3A). Only when compound (EtHg and MeHg) concentrations rose to 5 μM, did the oxidation of Trx1 increase significantly. In line with the fact that EtHg is the most toxic Hg compound to SH-SY5Y cells and in agreement with previous findings [10], EtHg-mediated Trx1 oxidation was visible as soon as after 6 h of exposure. On the other hand, Hg^{2+} did not produce any significant change in the oxidation state of Trx1, which could explain its lower toxicity for SH-SY5Y cells. Mitochondrial Trx2 is less susceptible to Hg compounds but its oxidation results in dimerization of Prx3, which reduces the antioxidant capacity of cells [38].

Pre-treatment of SH-SY5Y cells with BSO resulted in a dramatic reduction of intracellular GSH (−75%) and in a shift of the redox state

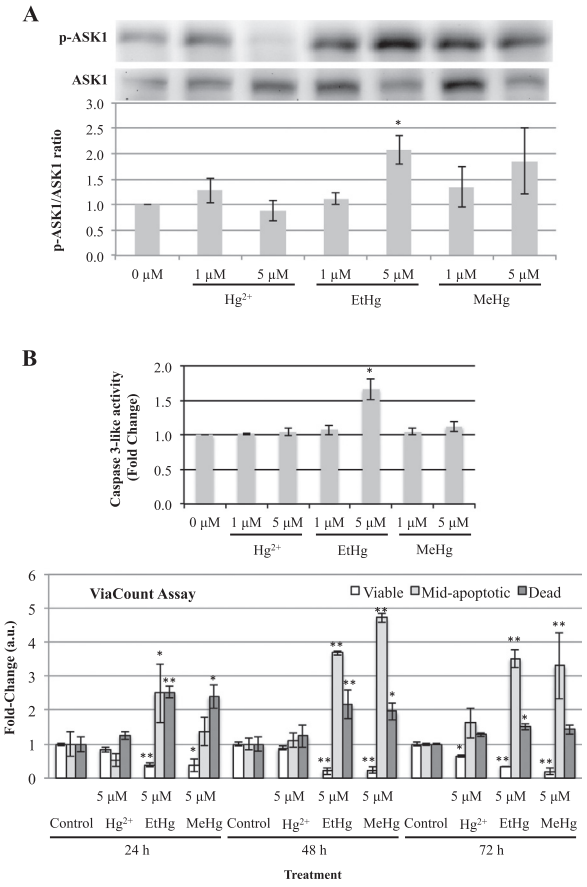

Fig. 6. Activation of ASK-1, Caspase-3 activity and apoptotic cell count in SH-SY5Y cells exposed to Hg compounds. Following exposure to mercury compounds for 24 h, SH-SY5Y cells were analyzed by Western blot to quantify activation of ASK-1 by the phosphorylation of Thr838 (pASK-1), and caspase-3-like activity was measured at 405 nm using a fluorimetric assay. The number of apoptotic cells following 24, 48 and 72 h of treatment with 5 μM of each mercurial was determined by the Guava ViaCount assay. Data in graphs are representative of 3 independent experiments (see Supplementary material Fig. S3 for further details). The blot concerning pASK-1 phosphorylation was chosen for better representing increased phosphorylation upon treatment with 5 μM EtHg ** $p < 0.01$ * $p < 0.05$ from control.

of Trx from mostly reduced to mostly oxidized, suggesting that the GSH system plays a role in protecting Trx from Hg toxicity. In fact, due to its high concentration in cells, GSH may act as a first target for reactive oxygen species (ROS) generated by Hg [18,39], and bind Hg compounds [15,17]. Most importantly, it can serve as a backup molecule for TrxR to keep Trx in a reduced state. The latter can be achieved either through direct reduction of Trx by GSH or indirectly via Grx [8]. Regardless of the pathway, it is clear by our results that depletion of GSH is directly linked to increased oxidation of Trx. In fact, in this cellular context, exposure to EtHg caused the highest oxidation of Trx and in parallel led to a very significant decrease in GSH levels. Most interestingly, exposure to Hg did not increase PSSG. During oxidative stress, increase in PSSG may function as a transitory protective mechanism to protect the integrity of critical Cys residues in proteins [40]. For example, Trx1 Cys73 has been shown to be glutathionylated during oxidative stress, which protects the enzyme from dimerization and allows latter reactivation by glutaredoxin [41]. By causing a decrease in reduced GSH levels, mercurials might hinder the formation of GSH-protein conjugates exposing critical cysteines to oxidative damage.

Glutaredoxin activity and expression was not significantly affected by exposure to mercurials, confirming previous observations in HeLa cells [3]. Thus, it is plausible to infer that Grx might play a role in keeping Trx functional when Hg downregulates TrxR. In fact, Grx can act as a backup for TrxR and thus keep Trx in a reduced state [23,25].

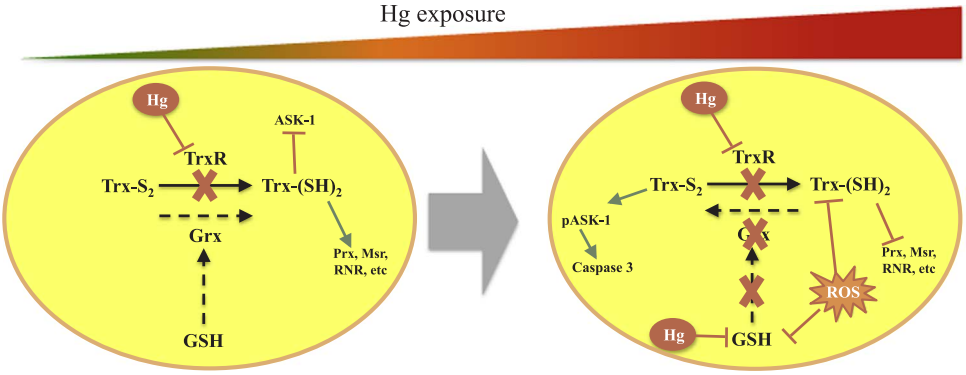

Fig. 7. Proposed mechanism of interaction between Hg compounds and Trx/GSH cross-talk leading to ASK-1 activation. When Hg levels are low, TrxR is the primary target. Thioredoxin activity is kept by a backup mechanism involving GSH/Grx that maintains Trx turnover from the oxidized to the reduced state, with GSH acting as the electron donor. This alternative pathway of Trx reduction allows the maintenance of downstream functions and keeps ASK-1 inactive. When Hg levels increase, so does GSH depletion. The decrease in available GSH hinders Grx activity, thus leading to Trx oxidation; accumulation of oxidized Trx blocks downstream functions and promotes the activation of the ASK-1 pathway leading to caspase activation and apoptosis. Redlines and X represent repressed activities or pathways; green arrows indicate active pathways; dashed arrows indicate the backup mechanism for Trx reduction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To confirm this hypothesis, we exposed primary cerebellar neurons from both wt and mGrx2D mice to EtHg. The dramatic increase in Trx2 oxidation in mGrx2D mice indicates that Grx2 is important in protecting Trx2 from oxidation caused by EtHg. A similar result was previously observed in HeLa cells subjected to Grx2 knock-down and exposed to TrxR inhibitors, 4-hydroxynonenal and Auranofin [25]. The physiological importance of Trx2 reduction by Grx2 is related to the fact that this enzyme is resistant to oxidation [42], more effective than GSH in reducing Trx2 in vitro [25] and that TrxR2 is not upregulated upon oxidative stress [27]. An equivalent role at the cytosol has been attributed to Grx1 [23], which besides acting as a backup for TrxR1, reducing the disulphide in the active site (Cys32 and Cys35) of Trx1, can also reduce the second disulphide between Cys62 and Cys 69 – which TrxR1 cannot – counteracting Trx1 overoxidation [8]. Curiously, this backup mechanism has been shown to work in the opposite direction, with TrxR being able to reduce the disulphide in the active site of the mitochondrial Grx 2 [24]. Moreover, Trx2 and Grx2 have also been demonstrated to reduce GSSG, which further stresses the importance of cross-talk between both systems [26].

To our best knowledge, these are the first results showing that Trx oxidation by Hg compounds is associated with increased phosphorylation of ASK-1 at Thr838 (Fig. 6A). Phosphorylation at this residue is known to engage ASK-1 activity [43] and the subsequent apoptotic pathway involving JNK, p38 and caspase-3 activation [44,45]. This is in agreement with previous results by Liu et al. [46], where p-38 activation was observed in human gastric cancer cells (SCM1) exposed to Thimerosal (ethylmercury thiosalicylate). Indeed, both Trx1 and Trx2 are known to complex with ASK-1 to repress its activity [47–49]. This inhibitory effect of Trx over ASK-1 is not dependent on its redox activity [49], but requires Trx Cys32 and Cys35 to be reduced. In contrast, oxidation of these residues promotes dissociation of the Trx-ASK-1 complex and phosphorylation of the latter [50]. Our results, for EtHg, support this hypothesis and show a remarkable agreement between Trx oxidation, ASK-1 phosphorylation, caspase-3 activation and increased number of apoptotic cells. Methylmercury appears to follow the same mechanism, even though delayed Trx oxidation (Fig. 3A) retards ASK-1 activation, caspase-3 engagement and increase in the number of apoptotic cells. In fact, caspase-3 mediated apoptosis has been previously reported to occur upon exposure to different Hg compounds [46,51–53]; thus, the mechanism suggested here for EtHg is likely to occur with other Hg compounds, albeit at differing paces that depend on the exposure concentration, magnitude of GSH depletion and Trx oxidation.

5. Conclusions

The findings here reported may be summarized as depicted in Fig. 7. At low Hg concentrations, TrxR will be the primary target to be affected leading to a significant decrease in its activity. A backup mechanism involving GSH/Grx comes into play, keeping Trx turnover from the oxidized to the reduced state, which enables downstream functions (e.g. Prx activity) and represses ASK-1 mediated cell death. As Hg levels increase, GSH becomes more oxidized. The decrease in available GSH hinders Grx activity and consequently Trx reduction, shifting the Trx pool towards the oxidized state. The accumulation of oxidized Trx may halt downstream functions, further increases ROS and promotes the activation of the ASK-1 pathway leading to caspase activation and apoptosis.

Overall, the results presented herein provide a valuable insight on the dynamics of mercury interaction with antioxidant systems and how it relates with the unfolding of cell death. Most importantly, this work links the effects of mercury compounds over the thioredoxin system with the effects over the glutathione system, providing a comprehensive view of the development of toxicity. It is also suggested for the first time that the ASK-1 pathway is relevant for Hg induced cytotoxicity.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.05.024.

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