Free Radical Research, Volume 38 Number 9 (September 2004), pp. 977–984

Taylor & Francis
healthsciences

In Vitro Interactions of Thallium with Components of the Glutathione-dependent Antioxidant Defence System

MARCELA S. VILLAVERDE, CECILIA E. HANZEL and SANDRA V. VERSTRAETEN*

Department of Biological Chemistry-IQUIFIB (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956 (C1113AAD), Buenos Aires, Argentina

Accepted by Professor A. Azzi

(Received 22 April 2004; In revised form 24 June 2004)

We investigated the hypothesis that thallium (Tl) interactions with the glutathione-dependent antioxidant defence system could contribute to the oxidative stress associated with Tl toxicity. Working *in vitro* with reduced glutathione (GSH), glutathione reductase (GR) or glutathione peroxidase (GPx) in solution, we studied the effects of Tl^{+} and Tl^{3+} (1–25 μM) on: (a) the amount of free GSH, investigating whether the metal binds to GSH and/or oxidizes it; (b) the activity of the enzyme GR, that catalyzes GSH regeneration; and (c) the enzyme GPx, that reduces hydroperoxide at expense of GSH oxidation. We found that, while Tl^{+} had no effect on GSH concentration, Tl^{3+} oxidized it. Both cations inhibited the reduction of GSSG by GR and the diaphorase activity of this enzyme. In addition, Tl^{3+} *per se* oxidized NADPH, the cofactor of GR. The effects of Tl on GPx activity depended on the metal charge: Tl^{+} inhibited GPx when cumene hydroperoxide (CuOOH) was the substrate, while Tl^{3+}-mediated GPx inhibition occurred with both substrates. The present results show that Tl interacts with all the components of GSH/GSSG antioxidant defence system. Alterations of this protective pathway could be partially responsible for the oxidative stress associated with Tl toxicity.

Keywords: Thallium (I); Thallium (III); Toxicity; Glutathione; Glutathione reductase; Glutathione peroxidase

Abbreviations: CuOOH, cumene hydroperoxide; DCI, 2,6-dichloroindophenol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); GSH, reduced glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; GPx, glutathione peroxidase

INTRODUCTION

Thallium (Tl) is a heavy metal present in the earth's crust as salts and minerals, at very low concentrations. It is widely used in the manufacture of electronic devices, in smelting operations, in cement factories, and in medical procedures.[1] This metal can be released to the environment,[2–5] exposing humans to its noxious effects. Tl has two oxidation states, thallous (Tl^{+}) and thallic (Tl^{3+}) cations. Tl^{3+} forms the corresponding hydroxide when dissolved in water,[6] decreasing the bioavailability of the metal. While several studies are focused on Tl^{+} toxicity (for review see[7,8]), there is limited information about Tl^{3+} effects on biological processes. Furthermore, Tl^{+} can be converted to Tl^{3+} by certain micro organisms,[9] which implies that the ingestion of these micro organisms can lead to Tl^{3+} poisoning.

Tl affects several tissues and systems, such as the epidermal, gastrointestinal, cardiovascular, and renal systems.[2,4,10] This metal crosses the blood–brain barrier,[11,12] and can deposit in the brain leading to neurodegeneration followed by secondary demyelination.[13,14] In addition, the co-localization of Tl deposits and the accumulation of end products of lipid oxidation in the brain of rats exposed to

*Corresponding author. Address: Departamento de Química Biológica, IQUIFIB (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina. Tel.: +54-11-4964-8287. Fax: +54-11-4962-5457. E-mail: verstraeten@ffyb.uba.ar

ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400000950

a chronic Tl-intoxication has been reported.[12] Since Tl has a high affinity for –SH groups, Aoyama *et al.*[15] related Tl-induced oxidative damage to cell components to a decrease in the reduced glutathione (GSH) concentrations. However, this relationship was not corroborated in other models of animal exposure to Tl.[16]

GSH is a tripeptide (Glu–Cys–Gly) that participates in a variety of biological processes.[17] Among other functions, GSH provides reduction equivalents for the maintenance of oxidant homeostasis,[18,19] participates in cell signalling,[20,21] and detoxifies drugs and xenobiotics, including toxic metals.[22–24] When the concentrations of certain oxidant species increases, two GSH molecules form a disulfide bridge through the oxidation of their cysteine –SH groups, leading to the formation of oxidized glutathione (GSSG). This reaction is catalyzed by the enzyme glutathione peroxidase (GPx), which has a key role in the scanvenging of H_2O_2 and organic peroxides.[17] In order to keep intracellular GSH/GSSG ratios within normal ranges (approximately 10/1),[17] GSH must be regenerated. The enzyme responsible for GSSG reduction is glutathione reductase (GR), which uses NADPH as a source of reduction equivalents.

The aim of the present work was to study *in vitro* the effects of Tl on the different components of the GSH-dependent antioxidant defence system. With this purpose, and working with the purified compounds, we investigated whether Tl^+ and/or Tl^{3+} (1–25 μM) affected: (a) the concentration of GSH, either by metal chelation and/or GSH oxidation; (b) the activity of the enzyme GR, and (c) the activity of GPx. While Tl^+ had no effect on GSH concentration, Tl^{3+} oxidized GSH. In addition, Tl^+ and Tl^{3+} inhibited GR activity in a concentration-dependent manner, as well as GR diaphorase activity. Finally, Tl^{3+}, but not Tl^+, inhibited GPx-mediated cumene hydroperoxide (CuOOH) reduction. However, using H_2O_2 as the substrate, GPx was inhibited by both Tl^+ and Tl^{3+}. Results indicate that Tl affects the GSH/GSSG system at multiple levels, reducing the amount of GSH available to detoxify oxidant species, interacting with GR and GPx cofactors and substrates, and directly inhibiting GR and GPx.

MATERIALS AND METHODS

Chemicals

Thallium (I) nitrate was obtained from Fluka (Milwaukee, WI). Thallium (III) nitrate was from Alfa Æsar (Ward Hill, MA). Glutathione (GSH), glutathione disulfide (GSSG), glutathione reductase (EC 1.6.4.2, GR), glutathione peroxidase (EC 1.11.1.9, GPx), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), sorbitol, xylenol orange, 2,6-dichloro-indophenol (DCI), NADPH, cumene hydroperoxide (CuOOH), and ferrous sulphate were from Sigma (St Louis, MO).

Tl Solutions

Tl^+ and Tl^{3+} stock solutions were prepared immediately before use. Tl (I) and Tl (III) were dissolved in Milli-Q water (pH ~ 5). Tl^{3+} stock solution was acidified with HCl 3 M to avoid the formation of $Tl(OH)_3$. The volume of acidic Tl^{3+} stock solution used for the experiments did not alter the pH of the 20 mM *Tris*–HCl buffer solution (pH 7.4) used in the different experiments.

Evaluation of Tl–GSH Interaction

GSH (50 μM) in 20 mM *Tris* (pH 7.4) buffer was added with increasing amount of either Tl^+ or Tl^{3+} (1–25 μM). After 20 min of incubation at 37°C, the concentrations of GSH and/or GSSG were quantified by (a) a colorimetric method and (b) HPLC with electrochemical detection.

(a) GSH Colorimetric Determination

After incubation, the reaction mixture (1 ml) was added with 1 ml of DTNB 2 mM in $NaHCO_3$ 5%[25] and further incubated for 10 min at 25°C. The absorbance of the samples were measured at 412 nm in a Shimadzu UV-265 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and the concentration of GSH in the samples were calculated from a calibration curve run in parallel. Results are expressed as the difference between the initial concentration of GSH and that measured after incubation in the presence of Tl, and indicated as GSH consumed.

(b) GSH and GSSG Determination by HPLC

After incubation, the reaction was stopped by the addition of 1 ml of perchloric acid 2 M and centrifuged at 29,000*g* for 20 min at 4°C. Aliquots containing 0.4 ml of the supernatant were kept at −80°C until use. GSH and GSSG were determined by HPLC[26] on a Supelcosil LC-18 (Supelco, Bellafonte, PA) column with an in-line BAS LC4C electrochemical amperometric detector with a glassy-carbon working electrode at an applied oxidation potential of 0.8 V. The GSH and GSSG concentration in the samples were calculated from a standard curve run in parallel. Results are expressed as the ratio GSSG/GSH.

Determination of GR Activity and GR Diaphorase Activity

GR (50 mU) in 20 mM *Tris* (pH 7.4) buffer was incubated for 5 min at room temperature in the presence of variable amounts of either Tl^+ or Tl^{3+} (1–25 μM). After incubation, 50 μM of GSSG and 60 μM of NADPH were added. The kinetics of NADPH consumption was followed from the decay of the absorbance at 340 nm.[25]

GR diaphorase activity was measured using the technique described by Gutierrez-Correa and Stoppani[27] with minor modifications. Aliquots of 0.2 ml of 20 mM *Tris* (pH 7.4) buffer containing 50 mU GR were placed in a 96 well microtiter plate, and incubated for 5 min either in the presence of variable amounts of Tl^+ or Tl^{3+} (1–25 μM). Samples were added with 85 μM NADPH and 35 μM DCI, and further incubated for 30 min at room temperature. After incubation, the absorbance at 600 nm was measured in a Microplate Reader Model 550 (BioRad, Tokyo, Japan).

Determination of GPx Activity

GPx activity was determined using two different protocols, measuring the GPx-mediated consumption of (a) NADPH,[28] and (b) hydroperoxide (CuOOH and H_2O_2).[29]

(a) NAPDH Consumption by GPx

GPx (50 mU) in 1 ml of 20 mM *Tris* (pH 7.4) buffer was incubated for 5 min at 25°C in the presence of variable concentrations of either Tl^+ or Tl^{3+} (1–25 μM). After incubation, 50 μM GSH, 55 mM CuOOH, 50 mU GR, and 40 μM NADPH were added to the samples. NADPH consumption was calculated from the decrease in the absorbance at 340 nm at 25°C.

(b) Hydroperoxide Consumption by GPx

GPx (50 mU) in 50 μl of 20 mM *Tris* (pH 7.4) buffer was placed in a 96 well microtiter plate, and incubated for 5 min at 25°C in the presence of variable concentrations of Tl^+ or Tl^{3+} (1–25 μM). After incubation, 5 μl of 0.5 mM GSH and 5 μl of 0.5 mM CuOOH or H_2O_2 were added, and samples were further incubated for 60 min. The remaining amount of CuOOH or H_2O_2 was measured as described by Dringen *et al.*[29] Briefly, the reaction was stopped by addition of 0.1 ml 25 mM H_2SO_4, and added with 0.1 ml of a solution containing 0.2 mM sorbitol, 0.5 mM ferrous sulphate, and 2 mM xylenol orange in 25 mM H_2SO_4. After 45 min incubation at room temperature, the absorbance at 570 nm was measured. The concentration of CuOOH or H_2O_2 in the samples were calculated from a calibration curve run in parallel.

Statistics

One-way analysis of variance (ANOVA test) followed by Fisher's test was performed using the routines available in StatView 5.0 (SAS Institute Inc., Cary, NC). Two-way ANOVA test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA). A probability (*P*) value lower than 0.05 was considered as statistically significant.

RESULTS

Evaluation of Tl Interaction with GSH

The possibility that Tl could interact with GSH was investigated by incubating GSH (50 μM) at 37°C for 30 min in the presence of Tl^+ or Tl^{3+} (1–25 μM) and measuring the concentration of GSH after incubation by reaction with DTNB. In the range of concentrations tested, Tl^+ slightly consumed GSH (Fig. 1A); for example, at 25 μM, Tl^+ only decreased the initial

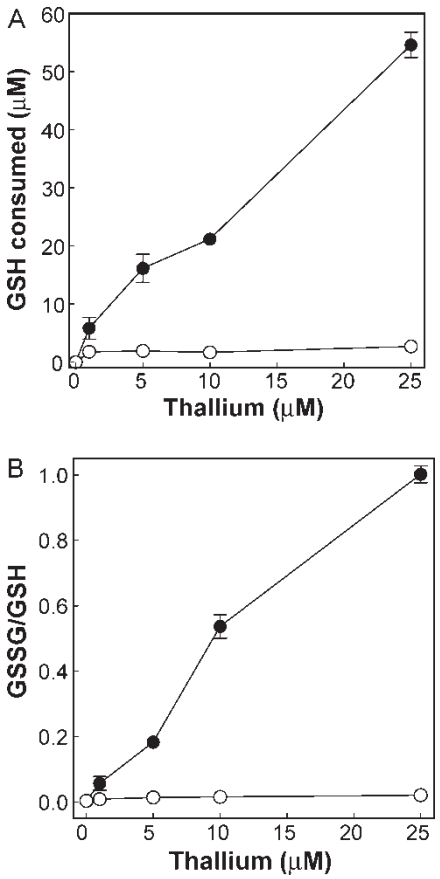

FIGURE 1 Effect of Tl on GSH oxidation. GSH (50 μM) in 20 mM *Tris*–HCl buffer (pH 7.4) was incubated in the absence or presence of Tl^+ (○), or Tl^{3+} (●) (1–25 μM). After incubation (A) remnant GSH was quantified by reaction with DTNB, and (B) GSH and GSSG concentrations were measured by HPLC with electrochemical detection, as described under "Materials and methods" section. Results are shown as the mean ± SEM of four independent experiments.

concentration of GSH in 2.7 μM ($P < 0.05$). On the contrary, Tl^{3+} (1–25 μM) caused a significant ($P < 0.005$) and concentration-dependent decrease of GSH concentration (Fig. 1A).

To evaluate whether the decrease in GSH concentration was due to its oxidation, the reaction mixture obtained after GSH (50 μM) incubation in the presence of Tl was also resolved by HPLC. In the chromatograms, only two peaks appeared that corresponded to GSH and GSSG, with no evidence of a Tl–GSH complex at the pH of the running (pH 2.7). Tl^{+} (1–25 μM) had no effect on GSSG/GSH ratio (Fig. 1B). Although at 25 μM Tl^{+}, a small amount of GSSG was found it was not statistically significant. Tl^{3+} increased GSSG/GSH ratio in a concentration (5–25 μM)-dependent manner ($P < 0.005$). For example, at 25 μM concentration the increase in GSSG/GSH ratio measured for Tl^{3+} was 48-times higher than that observed for Tl^{+}.

Interactions of Tl with GR

To investigate whether Tl could interfere with the normal GR activity, the reduction of GSSG by GR in the presence of NADPH was investigated. In our system and in the absence of additions, NADPH was oxidized at a rate of $5.3 \pm 0.4\ \mu M\ min^{-1}$. The presence of 1 μM Tl^{+} caused a 25% decrease ($P < 0.05$) in GR activity (Fig. 2A). The magnitude of the inhibition was similar at 1, 5 and 10 μM Tl^{+}, and increased at 25 μM Tl^{+}.

The possibility that Tl *per se* could oxidize NADPH was evaluated. NADPH (60 μM) was incubated in the presence of either Tl^{+} or Tl^{3+} (1–25 μM), and NADPH oxidation was measured. While Tl^{+} did not affect the amount of NADPH in the samples (Inset to Fig. 2B), Tl^{3+} significantly decreased NADPH concentration (Inset to Fig. 2B) with a rate constant of 0.15 ± 0.01 nmol NADPH min^{-1} (nmol Tl^{3+})$^{-1}$. The time necessary to reach the maximal Tl^{3+} (1–25 μM)-mediated NADPH oxidation was 30 min. At this point, NADPH was replenished and no further consumption of NADPH due to Tl^{3+} was observed (data not shown).

Taking these results into account, the effect of Tl^{3+} on GR activity was evaluated using two different protocols. First, the enzyme activity was measured after 5 min of incubation in the presence of Tl^{3+} (1–25 μM). Under this condition, Tl^{3+} caused a concentration-dependent decrease in GR activity (Fig. 2B), and the effect in magnitude resembled that obtained for Tl^{+}. Second, the activity was measured after 5 min incubation of GR in the presence of Tl^{3+} (1–25 μM) followed by a 30 min incubation in the presence of 60 μM NADPH to eliminate the effect of free Tl^{3+} on NADPH during the measurement of GR activity. After NADPH replenishment, a more dramatic decrease in GR activity due to Tl^{3+} was observed (Fig. 2B). The inhibitory effect of Tl^{3+} after 30 min of incubation was significantly higher than that observed after a 5 min incubation ($P < 0.005$, two-way ANOVA) reaching the highest effect at 10 μM Tl^{3+} concentration.

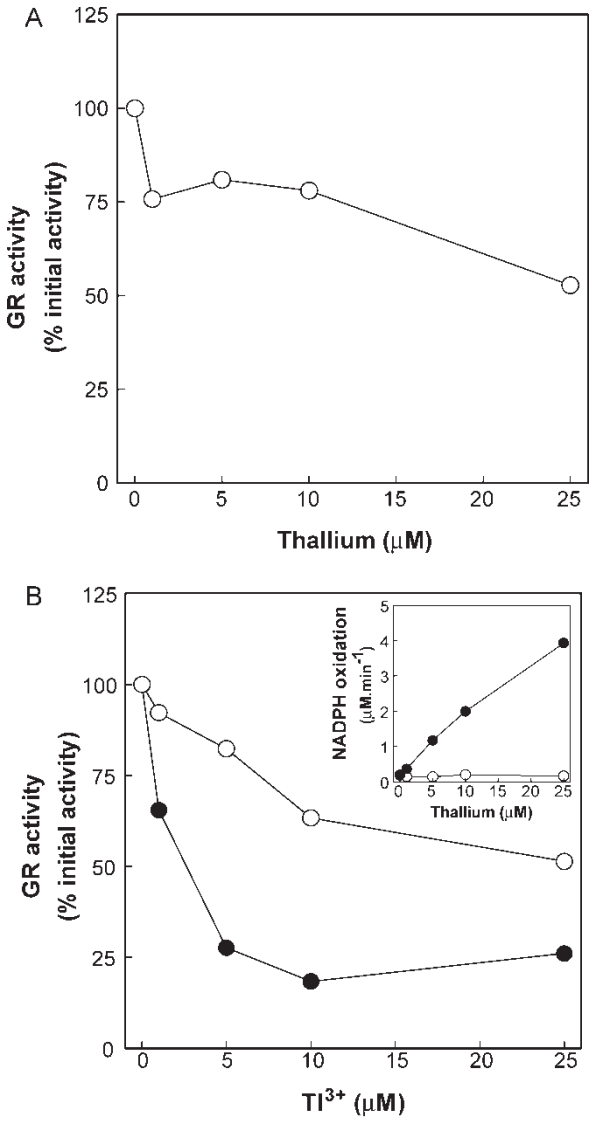

FIGURE 2 Effect of Tl on GR activity. GR (50 mU) in 20 mM *Tris*–HCl buffer (pH 7.4) was incubated for 5 min at 25°C either in the absence or presence of (A) Tl^{+} (○) (1–25 μM), and GR activity was measured as described under "Materials and methods" section. (B) GR was incubated in the presence Tl^{3+} (1–25 μM) for 5 min (○), or for 30 min followed by the addition of 60 μM NADPH (●), and after incubation the enzyme activity was measured. *Inset*: NADPH oxidation by Tl^{3+}. NADPH (60 μM) in 20 mM *Tris* buffer (pH 7.4) was incubated in the presence of either Tl^{+} (○) or Tl^{3+} (●) (1–25 μM) and the oxidation of NADPH was followed at 340 nm as described under "Materials and methods" section. Results are shown as mean ± SEM of four independent experiments.

The effect of Tl on GR diaphorase activity was investigated measuring the reduction of the dye 2,6-dichloro indophenol (DCI). Experiments were performed using lower metal/enzyme ratios (0.1 to 3 nmol Tl/pmol GR) than those used for GR activity

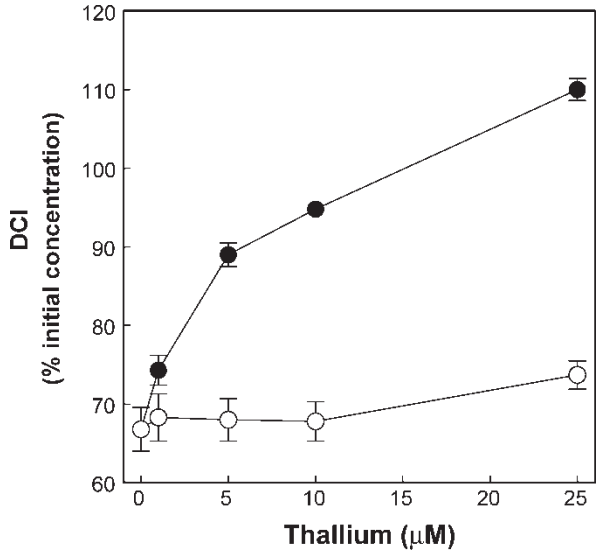

FIGURE 3 Effect of Tl on GR diaphorase activity. GR (50 mU) in 20 mM *Tris*–HCl buffer (pH 7.4) was incubated for 5 min at 25°C either in the absence or presence of Tl^{+} (○), or Tl^{3+} (●) (1–25 μM). GR diaphorase activity was measured as described under "Materials and methods" section. Results are expressed as the percentage of the initial DCI concentration, and are shown as mean ± SEM of four independent experiments.

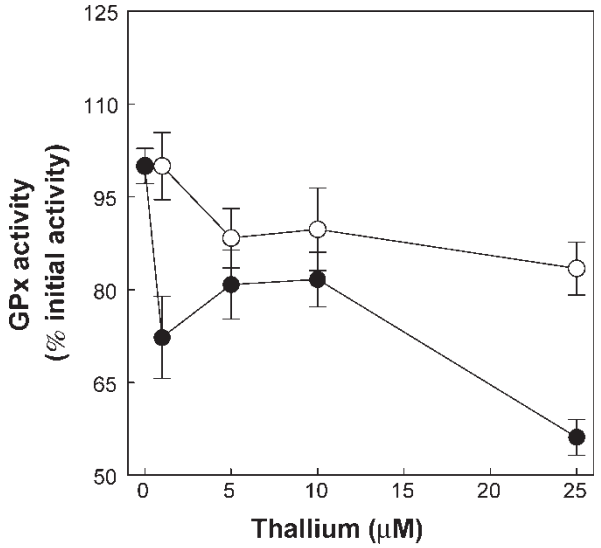

FIGURE 4 Effect of Tl on GPx-mediated NADPH oxidation. GPx (50 mU) in 20 mM *Tris*–HCl buffer (pH 7.4) was incubated for 5 min either in the absence or presence of Tl^{+} (○), or Tl^{3+} (●) (1–25 μM). After incubation, GPx activity was measured from NADPH oxidation as described under "Materials and methods" section. Results are shown as the mean ± SEM of four independent experiments.

measurement (0.6 to 15 nmol Tl/pmol GR), and in the absence of GSSG addition. In the absence of any additions, GR reduced 33 ± 1% of DCI after 30 min incubation. The addition of Tl^{+} (1–25 μM) caused a slight inhibition of the diaphorase activity, effect that was significant ($P < 0.05$) only at the highest concentration assessed (Fig. 3). Tl^{3+} decreased DCI reduction in a concentration-dependent manner, effect that was significant even at the lowest concentration tested ($P < 0.001$). When GR diaphorase activity was determined after 30 min of incubation in the presence of Tl^{3+} (1–25 μM) followed by NADPH replenishment, a significant and concentration-dependent decrease in enzyme activity was observed (data not shown). The inhibitory effect of Tl^{3+} after 30 min of incubation was significantly higher ($P < 0.001$) along the range of concentration tested (two-way ANOVA) respect to the values obtained after 5 min of incubation in similar conditions.

Evaluation of Tl Interaction with GPx

Tl effect on GPx activity was studied using two different methods. The first was based on the measurement of NADPH oxidation in a GR/GPx coupled system. The second method exclusively involved GPx and quantified the amount of substrate not being reduced by the enzyme.

In the absence of additions, GPx oxidized NADPH at a rate of $2.4 \pm 0.1\ \mu M\,min^{-1}$. The presence of Tl^{+} decreased GPx activity in a concentration (1–25 μM)-dependent manner (Fig. 4), although the effect was statistically significant ($P < 0.01$) only at 25 μM Tl^{+}. Tl^{3+} significantly inhibited the system ($P < 0.005$) along all the range of concentrations tested (Fig. 4).

When GPx-mediated hydroperoxide reduction was evaluated, different results were obtained for both metal species investigated. Tl^{+} (1–25 μM) did not affect CuOOH concentration (Fig. 5A) although caused a significant ($P < 0.005$) and concentration-dependent inhibition of H_2O_2 reduction by the enzyme (Fig. 5B). On the other hand, Tl^{3+} (10–25 μM) significantly ($P < 0.01$) inhibited GPx regardless the substrate used (Fig. 5A and B).

DISCUSSION

It has been described that Tl-intoxication leads to alterations of a considerable number of biological processes. Among others, it has been reported that Tl caused an increase in lipid oxidation products in five different brain regions of rats exposed to Tl^{+}, which magnitude was associated with the amount of metal deposited.[12] However, the mechanisms that participate in Tl-mediated oxidative stress are still not elucidated. Recently, we demonstrated that *in vitro* Tl^{+} and Tl^{3+} altered certain membrane physical properties[30] that could make the bilayer more prone to be oxidized.[31–33] Moreover, Tl toxicity is partially determined by its affinity to amino-, imino- and sulfhydryl- groups.[7] Given the importance of glutathione in the context of the cell antioxidant defence system,[17] the interaction of Tl with this

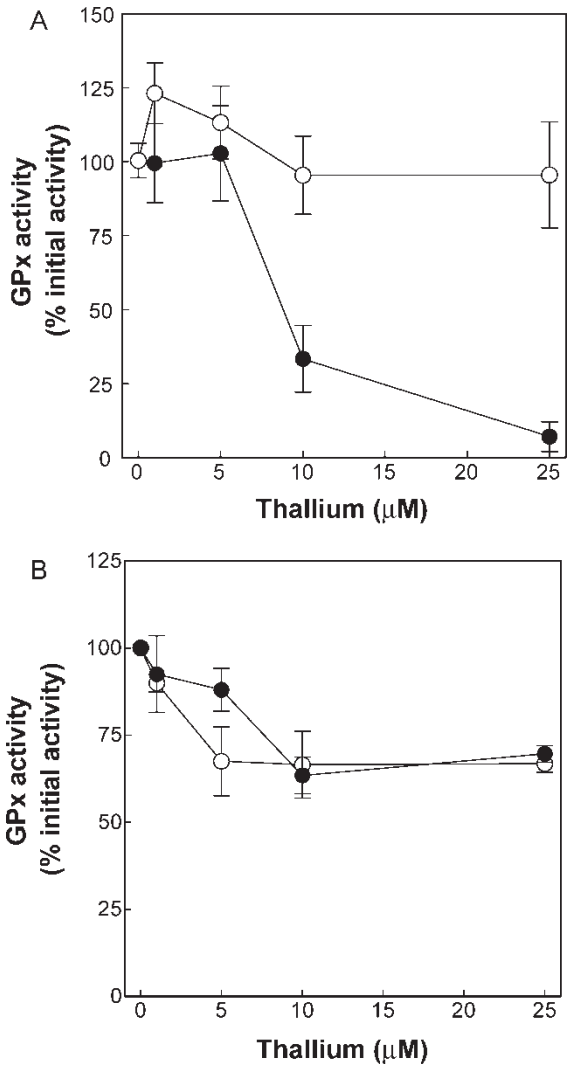

FIGURE 5 Effect of Tl on GPx-mediated hydroperoxide reduction. GPx (50 mU) in 20 mM *Tris*–HCl buffer (pH 7.4) was incubated for 5 min either in the absence or presence of Tl^+ (○), or Tl^{3+} (●) (1–25 μM). After incubation, 0.5 mM of (A) cumene hydroperoxide (CuOOH), or (B) H_2O_2 were added, and the samples were further incubated for 60 min. The amount of CuOOH or H_2O_2 remaining in the samples was measured as described under "Materials and methods" section. Results are shown as the mean ± SEM of at least four independent experiments.

molecule and/or with the enzymes involved in its use and regeneration, could lead to oxidative stress.

Until now, whether or not Tl affect glutathione metabolism has not been clearly established. Appenroth and Winnefeld[16] reported that the concentration of GSH in the kidney remained unchanged in Tl_2SO_4-mediated nephrotoxicity in rats. On the contrary, the administration of a single dose of Tl^+ malonate also to rats caused a decrease in kidney GSH concentration.[15] The Tl-induced reduction in tissue GSH could be due to a direct effect of Tl on the GSH/GSSG system and/or could be secondary to an increase in the amount of oxidant species generated by other mechanisms. In order to understand which components of GSH-dependent antioxidant defence system could be the targets of Tl-mediated damage, we worked *in vitro* with purified compounds.

Since Tl has a high affinity for –SH groups,[7] this metal could react with GSH, thus reducing the effective GSH concentration. In our system, no changes in GSH concentration due to Tl^+ were observed. The lack of a Tl^+–GSH complex formation was unexpected since at pH > 8, Tl^+ complexes free Cys[34] and GSH detection with DTNB was carried out at pH 10. On the other hand, the trivalent cation effectively decreased the amount of GSH. In the case of Tl^{3+}, the decrease in GSH concentration was at the expense of its oxidation. For example, at 25 μM Tl^{3+} the amount of GSH was reduced in a 50%. It has been described that Tl^{3+} rapidly forms the corresponding hydroxide when dissolved in water. Supporting that, Ralph and Twiss[35] calculated that in a 10^{-8} M Tl^{3+} aqueous solution, only 2×10^{-12} M is present as a free cation, and the remaining is present as $Tl(OH)_3$, among other compounds. The rapid interaction between Tl^{3+} and a molecule/s with metal-binding capacity, such as GSH, could prevent $Tl(OH)_3$ formation, thus increasing the relative amount of Tl^{3+} capable to oxidize GSH.

The second component of this system is GR, an enzyme formed by two subunits, each one carrying a single molecule of FAD at its active site. NADPH reduces FAD, which in turn reduces a disulfide bridge formed by two cysteine residues, and becomes available to interact with GSSG.[17] When Tl is present in the medium, the metal could potentially interact with the enzyme at two levels: reacting with its –SH groups, and/or with the FAD-containing domain that bears the diaphorase activity. The alteration of one or both activities of the enzyme could lead to an overall decrease in GR functionality. Tl^+ exclusively affected GR activity but did not alter the diaphorase activity indicating that Tl^+ may interact either with the Cys at the catalytic site, or with the aminoacid residues in its surroundings, while it has a poor or no interaction with the FAD domain. Since the reduction of Tl^+ to Tl^0 does not seem to occur *in vivo*, it is unlikely that a direct oxidation of the aminoacid residues in the catalytic region occurs; rather the binding of the cation is involved. On the other hand, Tl^{3+} altered both activities of the enzyme. This is a potent oxidant specie ($Tl^{3+}/Tl^+ \varepsilon^0$: 1.25 V) that could not only bind to certain aminoacid residues, but also could oxidize them. In fact, working with aqueous solutions of Tyr, we observed that Tl^{3+} (1–25 μM) induced the formation of dityrosine as evaluated from the appearance of its characteristic fluorescence spectrum (data not shown). Specifically, the oxidation by redox-active metals of Tyr_{114} located in the catalytic site of GR has been described to be partially responsible for the loss of activity of this enzyme.[27]

Interestingly, in addition to the inhibition of GR activity, Tl^{3+} also oxidized *per se* NADPH, the enzyme's cofactor. *In vivo*, the oxidation of NADPH by Tl^{3+} could have profound consequences on cell metabolism. A decrease in intracellular NADPH content could diminish the amount of GSH recycled through the reduction of GSSG by GR, and lead to an oxidant/antioxidant imbalance. In addition, when the amount of NADPH decreases, the hexose monophosphate pathway is stimulated in order to regenerate NADPH, increasing the consumption of glucose not associated with ATP production. The enhancement of this metabolic pathway secondary to GSH depletion was observed in a number of metabolic disorders, such as β-talassemia.[36]

Finally, the last component of GSH/GSSG system is the enzyme GPx. Using the traditional method for GPx determination[28] we found that Tl^{3+} and Tl^{+} decreased the activity of the enzyme. However, it is important to consider that the reaction mixture contained, besides Tl, GSH, GR, NADPH, GPx and hydroperoxide. In this work we presented experimental evidence showing that Tl decreases the amount of GSH (Tl^{3+}), inhibits GR activity (Tl^{+} and Tl^{3+}), and oxidizes NADPH (Tl^{3+}). Therefore, from those experiments whether the decrease in GPx activity is due to a lower enzyme activity or is a consequence of Tl interactions with one or more components of this system could not be established. Therefore, we evaluated the effect of Tl on GPx activity using a different method that measures the amount of hydroperoxide reduced by GPx. Interestingly, both metal species studied had a different behavior on GPx-mediated hydroperoxide reduction. Tl^{+} had no effect on CuOOH reduction although it caused a significant decrease in GPx activity when H_2O_2 was the substrate. Tl^{3+} affected GPx activity with no distinction between substrates. Considering the fact that both substrates were in a 20-fold excess respect to the highest Tl concentration used, a direct interaction of Tl with the hydroperoxide that could limit their accessibility to the enzyme can be discarded.

The active site of GPx contains a –SH adjacent to the selenol group[37,38] and both functional groups can be oxidized leading to the loss of activity.[39] This could be particularly true for Tl^{3+} which has a high redox potential, causing a net decrease in GPx activity. In the case of Tl^{+}, this metal does not have oxidant capacity but could be chelated by the selenol group itself, or by the aminoacid residues close to the catalytic site of the enzyme. The molecular mechanism by which Tl^{+} specifically affected H_2O_2 reduction requires further investigation.

Taken together, the present experimental evidence demonstrates that Tl affects glutathione-dependent antioxidant defence system, at least, at four levels (Fig. 6). This metal reduces the amount of GSH mainly due to its oxidation, although this mechanism could have the lowest impact on cell metabolism given the relative high concentrations of GSH in the cytosol. In normal conditions, GSH levels should be restored by the action of GR, which is impaired by Tl. This metal also limits the detoxification of hydroperoxide through the inhibition of the enzyme GPx. And finally, Tl^{3+} reduces the amount of NADPH, a cofactor of a large number of other oxido-reductase enzymes. Therefore, the decrease in NADPH concentration could activate the hexose monophosphate pathway that could lead to a depletion of cell glucose with severe consequences on cell metabolism. In conclusion, the impairment of GSH-mediated antioxidant defence system could partially account for the oxidative damage associated with Tl intoxication. Through this mechanism, Tl could cause its main symptoms of poisoning (alopecia, cardiomyopathy, nephrotoxicity and neurodegeneration), all these pathologies where misbalances in GSH metabolism that have been described.[40–44]

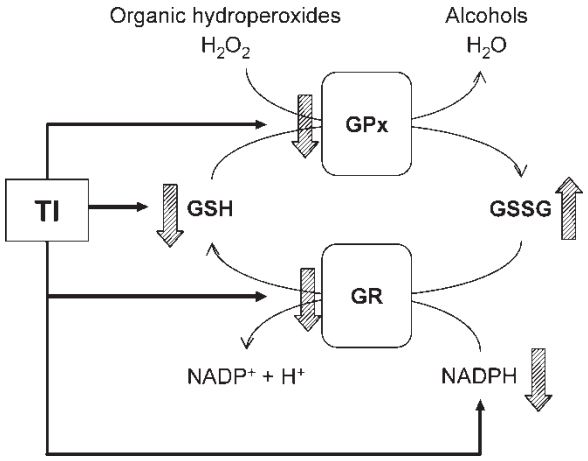

FIGURE 6 Scheme showing the postulated targets of Tl in glutathione-dependent antioxidant defence system.

Acknowledgements

This work was supported by grants from the Third World Academy of Sciences (01-350 RG/BIO/LA), the University of Buenos Aires (B 405) and the Agencia Nacional de Promoción Científica y Tecnológica (12285). The authors want to thank Dr Elena Cavanagh and Javier Ottaviani for their kind help in the determination of GSH/GSSG, and to Professor Patricia Oteiza for the helpful discussion of the work.

References

[1] ATSDR Thallium (1999) ATSDR (Agency for Toxic Substances and Disease Registry). Prepared by Clement International Corp., under contract 205-88-0608, Atlanta, GA.

[2] Heim, M., Wappelhorst, O. and Markert, B. (2002) "Thallium in terrestrial environments—Occurrence and effects", *Ecotoxicology* **11**, 369–377.
[3] Repetto, G., Del Peso, A. and Repetto, M. (1998) "Human thallium toxicity", *Thallium in the environment. Advances in environmental science and technology* (Wiley, USA), pp 167–199.
[4] Douglas, K.T., Bunni, M.A. and Baindur, S.R. (1990) "Thallium in biochemistry", *Int. J. Biochem.* **22**, 429–438.
[5] Xiao, T., Guha, J., Boyle, D., Liu, C.Q. and Chen, J. (2004) "Environmental concerns related to high thallium levels in soils and thallium uptake by plants in southwest Guizhou, China", *Sci. Total Environ.* **318**, 223–244.
[6] Lin, T.S. and Nriagu, J. (1998) "Revised hydrolysis constants for thallium(I) and thallium(III) and the environmental implications", *J. Air Waste Manag. Assoc.* **48**, 151–156.
[7] Leonard, A. and Gerber, G.B. (1997) "Mutagenicity, carcinogenicity and teratogenicity of thallium compounds", *Mutat. Res.* **387**, 47–53.
[8] Galvan-Arzate, S. and Santamaria, A. (1998) "Thallium toxicity", *Toxicol. Lett.* **99**, 1–13.
[9] Twining, B.S., Twiss, M.R. and Fisher, N.S. (2003) "Oxidation of thallium by freshwater plankton communities", *Environ. Sci. Technol.* **37**, 2720–2726.
[10] Moore, D., House, I., Dixon, A., Williams, G., Volans, G., Henry, J., Hughes, R.A.C., Cochrane, G.M. and Atkinson, P. (1993) "Thallium poisoning", *BMJ* **306**, 1527–1529.
[11] Galvan-Arzate, S. and Rios, C. (1994) "Thallium distribution in organs and brain regions of developing rats", *Toxicology* **90**, 63–69.
[12] Galván-Arzate, S., Martínez, A., Medina, E., Santamaría, A. and Ríos, C. (2000) "Subchronic administration of sublethal doses of thallium to rats: effects on distribution and lipid peroxidation in brain regions", *Toxicol. Lett.* **116**, 37–43.
[13] Bank, W.J., Pleasure, D.E., Suzuki, K., Nigro, M. and Katz, R. (1972) "Thallium poisoning", *Arch. Neurol.* **26**, 456–464.
[14] Davis, L.E., Standefer, J.C., Kornfeld, M., Abercrombie, D.M. and Butler, C. (1981) "Acute thallium poisoning: toxicological and morphological studies of the nervous system", *Ann. Neurol.* **10**, 38–44.
[15] Aoyama, H., Yoshida, M. and Yamamura, Y. (1988) "Induction of lipid peroxidation in tissues of thallous malonate-treated hamster", *Toxicology* **53**, 11–18.
[16] Appenroth, D. and Winnefeld, K. (1999) "Is thallium-induced nephrotoxicity in rats connected with riboflavin and/or GSH?—reconsideration of hypotheses on the mechanism of thallium toxicity", *J. Appl. Toxicol.* **19**, 61–66.
[17] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine, (Oxford University Press, London).
[18] Lou, M.F. (2003) "Redox regulation in the lens", *Progr. Ret. Eye Res.* **22**, 657–682.
[19] Meloni, M. and Nicolay, J.F. (2003) "Dynamic monitoring of glutathione redox status in UV-B irradiated reconstituted epidermis: effect of antioxidant activity on skin homeostasis", *Toxicol. In Vitro* **17**, 609–613.
[20] Morito, N., Yoh, K., Itoh, K., Hirayama, A., Koyama, A., Yamamoto, M. and Takahashi, S. (2003) "Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels", *Oncogene* **22**, 9275–9281.
[21] Canals, S., Casarejos, M.J., Bernardo, S., Solano, R.M. and Mena, M.A. (2003) "Selective and persistent activation of extracellular signal-regulated protein kinase by nitric oxide in glial cells induces neuronal degeneration in glutathione-depleted midbrain cultures", *Mol. Cell. Neurosci.* **24**, 1012–1026.
[22] Patrick, L. (2002) "Mercury toxicity and antioxidants: Part 1: role of glutathione and alpha-lipoic acid in the treatment of mercury toxicity", *Altern. Med. Rev.* **7**, 456–471.
[23] Johri, S., Shukla, S. and Sharma, P. (2002) "Role of chelating agents and antioxidants in beryllium induced toxicity", *Indian J. Exp. Biol.* **40**, 575–582.
[24] Chen, C.-J. and Liao, S.-L. (2003) "Zinc toxicity on neonatal cortical neurons: involvement of glutathione chelation", *J. Neurochem.* **85**, 443–453.
[25] Akerboom, T.P.M. and Sies, H. (1981) "Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples", *Methods Enzymol.* **77**, 373–382.
[26] Rodriguez-Ariza, A., Toribio, F. and López-Barea, J. (1994) "Rapid determination of glutathione status in fish liver using high-performance liquid chromatography and electrochemical detection", *J. Chromatogr. B* **656**, 311–318.
[27] Gutierrez-Correa, J. and Stoppani, A.O.M. (1997) "Inactivation of yeast glutathione reductase by Fenton systems: effect of metal chelators, catecholamines and thiol compounds", *Free. Radic. Res.* **27**, 543–555.
[28] Lawrence, R.A. and Burk, R.F. (1976) "Glutathione peroxidase activity in selenium-deficient rat liver", *Biochem. Biophys. Res. Commun.* **71**, 952–958.
[29] Dringen, R., Kussmaul, L. and Hamprecht, B. (1998) "Detoxification of exogenous hydrogen peroxide and organic hydroperoxides by cultured astroglial cells assessed by microtiter plate assay", *Brain Res. Protoc.* **2**, 223–228.
[30] Villaverde, M.S. and Verstraeten, S.V. (2003) "Effects of thallium(I) and thallium(III) on liposome membrane physical properties", *Arch. Biochem. Biophys.* **417**, 235–243.
[31] Verstraeten, S.V. and Oteiza, P.I. (1995) "Sc^{3+}, Ga^{3+}, In^{3+}, Y^{3+}, and Be^{2+} promote changes in membrane physical properties and facilitate Fe^{2+}-initiated lipid peroxidation", *Arch. Biochem. Biophys.* **322**, 284–290.
[32] Verstraeten, S.V., Nogueira, L.V., Schreier, S. and Oteiza, P.I. (1997) "Effect of trivalent metal ions on phase separation and membrane lipid packing: role in lipid peroxidation", *Arch. Biochem. Biophys.* **338**, 121–127.
[33] Verstraeten, S.V., Keen, C.L., Golub, M.S. and Oteiza, P.I. (1998) "Membrane composition can influence the rate of Al^{3+}-mediated lipid oxidation: effect of galactolipids", *Biochem. J.* **333**, 833–838.
[34] Garcia Bugarin, M., Casas, J.S., Sordo, J. and Filella, M. (1989) "Thallium (I) interactions in biological fluids: a potentiometric investigation of thallium(I) complex equilibria with some sulphur-containing amino acids", *J. Inorg. Biochem.* **35**, 95–105.
[35] Ralph, L. and Twiss, M.R. (2002) "Comparative toxicity of thallium(I), thallium(III), and cadmium(II) to the unicellular alga Chlorella isolated from Lake Erie", *Bull. Environ. Contam. Toxicol.* **68**, 261–268.
[36] Cappellini, M.D., Tavazzi, D., Duca, L., Graziadei, G., Mannu, F., Turrini, F., Arese, P. and Fiorelli, G. (1999) "Metabolic indicators of oxidative stress correlate with haemichrome attachment to membrane, band 3 aggregation and erythrophagocytosis in beta-thalassaemia intermedia", *Br. J. Haematol.* **104**, 504–512.
[37] Forstrom, J.W., Zakowski, J.J. and Tappel, A.L. (1978) "Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine", *Biochemistry* **17**, 2639–2644.
[38] Chaudiere, J., Wilhelmsen, E.C. and Tappel, A.L. (1984) "Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans", *J. Biol. Chem.* **259**, 1043–1050.
[39] Blum, J. and Fridovich, I. (1985) "Inactivation of glutathione peroxidase by superoxide radical", *Arch. Biochem. Biophys.* **240**, 500–508.
[40] Giralt, M., Cervello, I., Nogues, M.R., Puerto, A.M., Ortin, F., Argany, N. and Mallol, J. (1996) "Glutathione, glutathione S-transferase and reactive oxygen species of human scalp sebaceous glands in male pattern baldness", *J. Investig. Dermatol.* **107**, 154–158.
[41] Bartfay, W.J. and Bartfay, E. (2000) "Iron-overload cardiomyopathy: evidence for a free radical—mediated mechanism of injury and dysfunction in a murine model", *Biol. Res. Nurs.* **2**, 49–59.
[42] Kedziora-Kornatowska, K., Szram, S., Kornatowski, T., Szadujkis-Szadurski, L., Kedziora, J. and Bartosz, G. (2003) "Effect of vitamin E and vitamin C supplementation on antioxidative state and renal glomerular basement membrane thickness in diabetic kidney", *Nephron Exp. Nephrol.* **95**, 134–143.
[43] Bhatia, S., Shukla, R., Venkata Madhu, S., Kaur Gambhir, J. and Madhava Prabhu, K. (2003) "Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy", *Clin. Biochem.* **36**, 557–562.
[44] Martin, L.J., Brambrink, A.M., Price, A.C., Kaiser, A., Agnew, D.M., Ichord, R.N. and Traystman, R.J. (2000) "Neuronal death in newborn striatum after hypoxia-ischemia is necrosis and evolves with oxidative stress", *Neurobiol. Dis.* **7**, 169–191.