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Threshold protective effect of glutathione on inorganic mercury inhibition of cerebral sodium pump

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ABSTRACT

Glutathione (GSH) is a sulphydryl tripeptide that protects the oxidation of critical sulphydryl ligands in thiol-containing biological proteins. Consequently, the effect of GSH on the inhibition of cerebral sodium pump elicited by inorganic mercury was investigated. We observed that GSH prevented but could not relieve the binding of Hg^{2+} to sulphydryl groups at the adenosine triphosphate and cationic sites of the electrogenic pump. Furthermore, GSH (= 4 mM) markedly inhibited the activity of the pump. Since GSH is differentially distributed in various organelles (1-15mM), we speculate that there is a probable differential sensitivity of the sodium pump in various organelles towards the antioxidant tripeptide suggesting the possibility of a strong dynamics in the regulation of GSH in the various compartments of the organelles. Taken together, GSH may possibly be a potential candidate as a pre-intervention remedy in cases of mammalian exposure to inorganic mercury.

KEYWORDS

Inorganic mercury;
Sodium pump;
Thiols;
Glutathione;
Cerebral;
ATP site;
Cationic sites.

INTRODUCTION

Na^+/K^+-ATPase (EC 3.6.1.37) is an enzyme embedded in the cell membrane, responsible for the generation of the membrane potential in the central nervous system (CNS) necessary to maintain neuronal excitability[1]. Furthermore, it is of particular interest that Na^+/K^+-ATPase has been well documented as a sulfhydryl-containing enzyme and consequently could be sensitive to oxidizing agents[2,3]. In this regard, we have recently observed that this transmembrane enzyme is sensitive to agents that is capable of oxidizing thiols such as selenium containing compounds[4,5] as well as oxidative stress[6,7] and inorganic mercury[8]. In fact, it has been well established that inorganic mercury is capable of oxidizing thiols and also induces oxidative stress[9]. Generally, in our group and elsewhere, we have reported that thiol-containing enzymes, such as δ-aminolevulinic acid dehydratase and Na^+/K^+-ATPase are assumed to be a target for mercury toxicity[10-13].

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This property of inorganic mercury is due to the high bonding affinity between mercury and sulfur. Consequently, mercury has been found bound to metallothioneins and small molecular weight thiols such as cysteine[14] and GSH[15] and has also been secreted from liver cells into bile as a complex with GSH[16], which is the major non-protein thiol in liver[17,18].

Our group has carried out progressive study to demonstrate the location of endogenous thiols that are critical to the functioning of the sodium pump. In our first attempts[4,5], we observed that critical sulphydryl groups of the enzyme are located at the ATP binding sites. More recently, we further observed that the Na^+ and K^+ sites may also contain critical thiols contingent to the normal functioning of the pump[8]. Specifically, in these reports[5,8] we reported that diphenyl diselenide could inhibit the pump in the absence of ATP but not Na^+ and K^+, whereas on the other hand, inorganic mercury could inhibit the pump in the absence of both cations and nucleotides. Hence based on the molecular volumes of these compounds (diphenyl diselenide and Hg^{2+}) relative to the substrates of the pump (ATP, Na^+ and K^+) we concluded that diphenyl diselenide which have hitherto been demonstrated to be a potent thiol oxidant could not possibly reach the cationic sites due to its relatively large molecular volume, whereas Hg^{2+} with a relatively small molecular volume compared to the cations could reach these sites and consequently inhibit the enzyme. Of more importance though is the observation that the binding of mercury to endogenous thiols on this enzyme can be prevented by exogenous thiols like cysteine[8]. One worrisome puzzle is the fact that cysteine could not relieve the inhibition imposed by Hg^{2+} thereby necessitating the need for the evaluation of other thiols vis-à-vis their potentials to relieve the inhibition imposed by Hg^{2+}. This evaluation becomes highly imperative considering the fact that mercury causes irreversible damage to important macromolecules and that thiols have different degree of reactivity towards oxidizing agents[19,20]. In this regard, the evaluation of the relationship between glutathione and the activity of sodium pump becomes highly crucial considering the fact that it is ubiquitously and differentially distributed in various organelles of the cells[17,18,21-25]. Hence the present study was designed to investigate the effect of GSH on the inhibitory effect of inorganic mercury on the activity of the transmembrane protein Na^+/K^+-ATPase.

MATERIALS AND METHODS

Chemicals

Adenosine triphosphate (ATP), ouabain, GSH were obtained from Sigma-Aldrich. All other chemicals used were of analytical grade and obtained from FLUKA, BDH and other standard commercial suppliers.

Animals

Male adult Wistar rats (200-250 g) from our own breeding colony were used. Animals were kept in separate animal cages, on a 12-h light: 12-h dark cycle, at a room temperature of 22-24°C, and with free access to food and water. The animals were used according to standard guidelines on the Care and Use of Experimental Animal Resources.

Oxidation of glutathione

The rate of thiol oxidation was determined in the presence of 50 mM Tris–Cl, pH 7.4, and 50-150 μM of mercury chloride. The rate of thiol oxidation was evaluated by measuring the disappearance of –SH groups. Free –SH groups were determined according to Ellman[26]. Incubation at 37°C was initiated by the addition of the thiol compounds. Aliquots of the reaction mixture (100 μl) were checked for the amount of –SH groups at 412 nm after addition of the color reagent 5'5'-dithio-bis(2-nitrobenzoic) acid (DTNB).

Assay of Na^+/K^+-ATPase activity

Immediately after the animals were euthanized, the brain was removed and the homogenate was prepared in 0.05 M Tris-HCl, pH 7.4. The homogenate was centrifuged at 4,000 rpm at 4^0C for 7 min and the supernatant was used for the assay of Na^+/K^+-ATPase. The reaction mixture for Mg^{2+}-dependent-Na^+/K^+-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl, 200 mM sodium azide and 50 mM Tris–HCl, pH 7.4 and 100–180 μg of protein, in a final volume of 500 μl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+/K^+-ATPase activity was calculated by the difference between the two assays. Re-

leased inorganic phosphorous (Pi) was measured by the method of Fiske and Subbarow[27]. To check whether pre-incubation of homogenates without a cationic component of the assay medium will affect the interaction of Hg^{2+} with the Na^+/K^+-ATPase activity, Hg^{2+} and enzyme were incubated at 370C for 10 min, with the selective exclusion of each Mg^{2+}, Na^+, K^+ in the preincubating medium. All the experiments were conducted at least three times and similar results were obtained. Protein was measured by the method of Lowry *et al.*[28], using bovine serum albumin as standard.

Furthermore, we also investigated the possible effect of a GSH on the interaction of Hg^{2+} with sulphydryl groups located at the cationic as well as ATP binding sites of the sodium pump. In this case, GSH was either pre-incubated or post-incubated with enzyme and Hg^{2+} with the selective exclusion of either each of the nucleotide or cations necessary for the functioning of the sodium pump. Also the effect of varying concentrations of glutathione (0-40mM) on the activity of the transmembrane enzyme was also tested. For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid (TCA) were used to correct for nonenzymatic hydrolysis of substrates. Enzyme activity was expressed as nmol of phosphate (Pi) released min^{-1} mg $protein^{-1}$.

Statistical analysis

Results were analyzed by appropriate analysis of variance (ANOVA) and this is indicated in text of results. Duncan's Multiple Range Test was applied where appropriate. Differences between groups were considered to be significant when $P < 0.05$.

RESULTS

GSH vs Hg^{2+} vs thiols at nucleotide site on sodium pump

The inhibitory effect of Hg^{2+} on the activity of the ouabain-sensitive Na^+/K^+-ATPase is presented in Figure 1. Apparently, Hg^{2+} inhibited the activity of the Na^+/K^+-ATPase in a concentration dependent manner. One-way ANOVA reveal that the inhibitory effect of Hg^{2+} on the cerebral transmembrane enzyme is significant ($P<0.05$) at concentration = 10μM when compared to the control. Pre-incubation of GSH with the enzyme in the presence of mercury abolished the inhibitory effect of Hg^{2+} on the activity of the enzyme while post-incubation of the cerebral enzyme with GSH did not relieve the inhibition posed by mercury on the activity of the sodium pump. In a similar fashion, when Mg^{2+} was excluded from the pre-incubation medium, Hg^{2+} caused a significant concentration-dependent inhibition of ATP hydrolysis by the transmembrane transporter (data not shown). The essence of Mg^{2+} in the incubating medium is to provide stability to the ATP ($MgATP^{2-}$) molecule and consequently, the effect of Hg^{2+} on the normal function of the pump in the absence of both ATP and Mg^{2+} is similar.

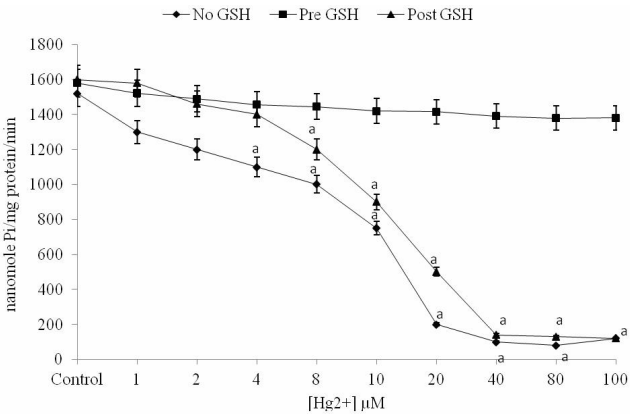

Figure 1 : Effect of Hg^{2+} on the activity of Na^+/K^+-ATPase. Reaction was started either by addition of ATP after 10 mins pre-incubation in the presence of Hg^{2+} (No GSH) or by addition of ATP after 10 mins pre-incubation in the presence of GSH and Hg^{2+} (Pre GSH) or by addition of ATP and GSH after 10 mins pre-incubation in the presence of Hg^{2+} (post GSH). Data are presented as mean ± SD of independent experiment carried out in different days. Data are analyzed using one-way ANOVA followed by Duncan test. [a]Represent significant difference from control at P< 0.05.

GSH vs Hg^{2+} vs thiols at cationic site on sodium pump

The inhibition of sodium pump by Hg^{2+} in the absence of Na^+ (Figure 2) is similar to that pattern of inhibition obtained when the reaction was initiated with ATP. Similarly, when GSH is present in the pre-incubating medium; there was no observed marked inhibition of the pump by Hg^{2+}. Conversely, introduction of GSH into the reaction medium after Hg^{2+} has been previ-

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ously introduced; neither revert nor relieve the inhibition imposed by Hg^{2+}. For all comparison, one-way ANOVA was employed and significant level was determined at $p < 0.05$. In the same vein, Figure 3 shows

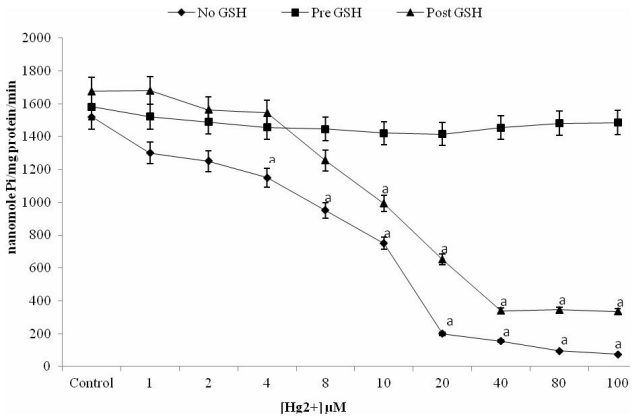

Figure 2 : Influence of Hg^{2+} on the activity of Na^+/K^+-ATPase upon the exclusion of Na^+ from the pre-incubation medium. Reaction was started either by addition of Na^+ after 10 mins preincubation in the presence of Hg^{2+} (No GSH) or by addition of Na^+ after 10 mins pre-incubation in the presence of 2 mM GSH and Hg^{2+} or by addition of Na^+ and 2 mM GSH after 10 mins preincubation in the presence of Hg^{2+}. Data is presented as mean ± SD of independent experiment carried out in different days. Data are analysed using one-way ANOVA followed by Duncan test. [a]Represent significant difference from control at P< 0.05

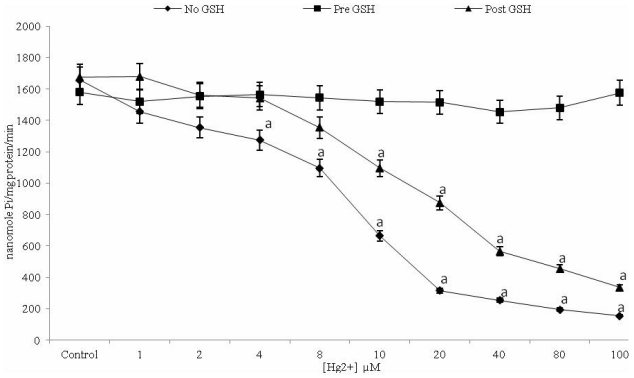

Figure 3 : Influence of Hg^{2+} on the activity of Na^+/K^+-ATPase upon the exclusion of K^+ from the pre-incubation medium. (No GSH) indicate that reaction was started by addition of K^+ after 10 mins pre-incubation in the presence of Hg^{2+}, whereas (Pre GSH) denotes reaction that was initiated by addition of K^+ after 10 mins pre-incubation in the presence of 2 mM GSH and Hg^{2+}. On the other hand (Post GSH) indicates reaction that was started by addition of K^+ and 2 mM GSH after 10 mins pre-incubation in the presence of and Hg^{2+}. In all cases, data is presented as mean ± SD of independent experiment carried out in different days. Data are analysed using one-way ANOVA followed by Duncan test. aRepresent significant difference from control at P< 0.05.

the effect of Hg^{2+} on the enzyme activity when K^+ was omitted from the preincubation medium. Similarly, this pattern of inhibition was similar to that obtained when either Na^+ or ATP was omitted from the pre-incubating medium. The inhibitory effect of Hg^{2+} was abolished when the reaction medium was preincubated with 2 mM GSH whereas the inhibition was not relieved when 2 mM GSH was post-incubated with the enzyme ($p<0.05$). As we would expect, Hg^{2+} caused a significant inhibition on the activity of transmembrane transporter in absence of all cations (Na^+, K^+ and Mg^{2+}) in a fashion similar to that obtained when the individual cations are excluded from the preincubating medium (data not shown). In fact, in the presence of all cations, Hg^{2+} exerted similar marked inhibitory effect on the activity of the sodium pump (data not shown). Either in the presence or absence of all cations, preincubating with GSH abolished the inhibitory effect of Hg^{2+} whereas post-incubation has no effect.

Effect of GSH on the activity of Na^+/K^+-ATPase

Figure 4 shows that GSH inhibits the activity of the ouabain-sensitive Na^+/K^+-ATPase in a concentration dependent manner. One-way ANOVA reveal that the inhibitory effect of GSH on the cerebral transmembrane enzyme is significant ($P<0.05$) at concentration =4mM when compared to the control.

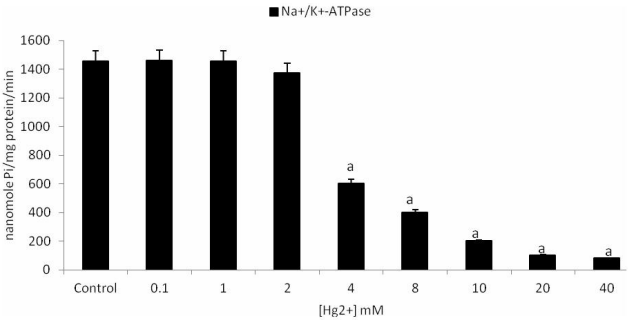

Figure 4 : Effect of different concentrations of GSH on the activity of Na^+/K^+-ATPase. Reaction was started by addition of ATP after 10 mins pre-incubation in the presence of GSH. Data is presented as mean ± SD of independent experiment carried out in different days. Data are analysed using one-way ANOVA followed by Duncan test. aRepresents significant difference from control at P< 0.05.

Effect of Hg^{2+} on oxidation of GSH

Figure 5 shows the effect of Hg^{2+} on the oxidation of GSH. The rate of GSH oxidation was accelerated by Hg^{2+}. In fact, the oxidation of GSH was increased

as a function of time and concentration of Hg^{2+}.

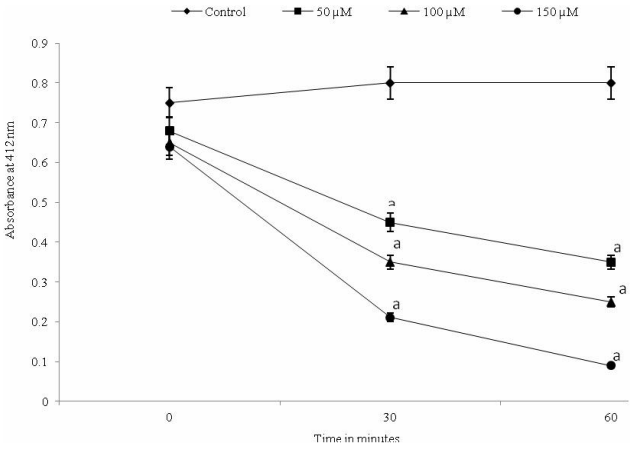

Figure 5 : Effects of different concentrations of Hg^{2+} on the rate of glutathione oxidation. The rate of oxidation was evaluated at the indicated times. Data are the means of three to four independent experiments carried out in different days. Data are expressed as mean ± SEM and post-hoc comparisons were done by Duncan´s multiple range test. Letters indicate significant difference in relation to the control ($p < 0.05$).

DISCUSSION

Generally, glutathione is ubiquitously distributed in organelles of eukaryotic cells[17,18] and provide intracellular defense against mercury-induced neurotoxicity[29-31], thus making GSH a candidate intervention strategy against mercury toxicity. Interestingly, our data show that GSH shields the interaction of Hg^{2+} with the sodium pump (Figures 1-3). Since Hg^{2+} oxidizes GSH (Figure 5) hence, we conclude that the exogenous GSH possibly has a sparing effect on oxidation of endogenous thiols of the transmembrane transporter by Hg^{2+}. Furthermore, the results obtained when ATP, Na^+, K^+, Mg^{2+} and all cations were used to initiated the reaction in the presence of only Hg^{2+} is in part confirmatory and further suggest that Hg^{2+} may be interacting with the cationic and nucleotide sites and that these sites must contain thiols necessary for the binding of these substrates as earlier demonstrated in our recent report[8]. The inability of GSH to relieve the inhibitory effect of Hg^{2+} on the activity of the transmembrane transporter (Figures 1-3) suggest that there is a possible strong affinity between the thiol residues on the enzyme's active sites and Hg^{2+} and this speculations is in conformity with earlier reports[8,11]. On the other hand, it is possible that steric factors which may affect the proximity of GSH to the bound Hg^{2+} may also play a role[19,20]. Of note however is the fact that GSH is differentially distributed in cellular compartments such as cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM)[32]. In view of the above, we tested the effect of increasing concentration of GSH on the activity of the cerebral pump under study. Figure 4 shows that at concentrations 4mM or greater, GSH also inhibited the activity of the sodium pump. While the mechanism of this inhibition is a subject of future research, it is worrisome that the inhibition of the pump by GSH is = 4mM since several authors have reported that GSH concentration range is 1-15mM in cellular compartments[17,18,21-25]. One possible speculation is that there is the possibility of variants of the sodium pump that may have differential tolerance to GSH *in vivo* or the levels of GSH may be kept far below the inhibitory level suggesting a possible high Km for the enzyme. Apparently, the dynamics and chemistry of GSH vis-à-vis the activity of the sodium pump can be complex and this complexity is obvious in the light of data earlier obtained in our laboratory in which we have consistently observed that treatment of rats or mice with diorganyl diselenides always increase the level of this important antioxidant molecule GSH with concomitant increase in the activity of the sodium pump. In fact, we equally observed that a decrease in the level of GSH is associated with decrease in the activity of the cerebral sodium pump[7,33]. Apparently then in vitro and in vivo data on the possible interaction of GSH with the sodium pump does not correlate and consequently, the use of GSH as a first line remedy in the management of inorganic mercury toxicity holds promise in view of the foregoing.

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