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The Role of Intracellular Glutathione in Inorganic Mercury-Induced Toxicity in Neuroblastoma Cells

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Abstract

It is well known that antioxidants containing sulfhydryl (–SH) groups are protective against the toxic effects of mercury. The current study was designed to elucidate the mechanism(s) of the cytoprotective effects of glutathione (GSH) and *N*-acetylcysteine (NAC) against the toxicity of inorganic mercury (HgCl₂) in neuroblastoma cells (N-2A). The obtained results demonstrated the protective effects of these compounds in a dose dependant manner up to 95 and 74% cell viability, respectively as compared to the control of HgCl₂ of 10%. The administration of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, increased the toxicity of HgCl₂ in a dose dependent manner. Moreover, BSO treatment attenuated the levels of the cellular free –SH concentrations at low concentrations (1–100 μM) of HgCl₂. The data also show that cellular thiol concentrations were augmented in the presence of GSH and NAC and these compounds were cytoprotective against HgCl₂ and this is due to up regulating of GSH synthesis. A reduction in intracellular levels of GSH was observed with treatment of HgCl₂. In addition, the ratio of GSH/GSSG increased from 16:1 to 50:1 from 1 to 10 μM concentration of HgCl₂. The ratio of GSH/GSSG then decreased from 4:1 to 0.5:1 with the increase of concentration of HgCl₂ between 100 μM and 1 mM due to the collapse of the N-2A cells. It was of interest to note that the synthesis of GSH was stimulated in cells exposed to low concentration of HgCl₂ when extra GSH is available. These data support the idea that the loss of GSH plays a contributing role to the toxic effects of HgCl₂ and that inorganic mercury adversely affects viability, through altering intracellular –SH concentrations. The data further indicate that the availability of GSH to the cells may not be sufficient to provide protection against mercury toxicity and the *de novo* synthesis of intracellular GSH is required to prevent the damaging effects of mercury.

Keywords

GSH; Synthesis; *N*-acetylcysteine; Cystine; GSSG

Introduction

Mercury (Hg) in the environment is an increasing health concern. In humans, mercury toxicity has been implicated in the development of various neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's diseases and Parkinson's disease [25]. Exposure to mercury, as a toxic substance, comes in different forms: elemental mercury, organic mercury and inorganic mercury [28]. Once elemental mercury is absorbed in the body, it is retained as inorganic mercury in the brain for several years [8]. Inorganic mercury (Hg²⁺) is known to be

formed by the demethylation of methyl mercury (MeHg) in the brain after long-term exposure to MeHg [30]. The levels of inorganic mercury (Hg^{2+}) are elevated in the cerebellum of Alzheimer's disease patients [15]. Moreover, HgCl_2 has been found to increase β amyloid secretion in SH-SY5Y neuroblastoma cells [27]. In addition, HgCl_2 was found to increase free radicals production in synaptosome preparations leading to oxidative damage [18]. Therefore, inorganic mercury is an important form of mercury that contributes significantly to this heavy metal neurotoxicity and neurodegeneration.

Inorganic mercury binds to various molecular weights of thiol-containing proteins (glutathione, cysteine, and albumin) [33] with high binding ability [34]. Mercury competes for thiol groups on the GSH molecule which forms GS-Hg-SG from GSH [12]. During injury or normal metabolism, glutathione plays a significant role in protecting the central nervous system against oxidative stress [1,13]. Reduced glutathione (GSH) contains reactive protein sulfahydryls [36] that can act as a direct antioxidant by its interaction with free radicals [11,29,31]. GSH can also perform xenobiotic function through detoxification by conjugation reactions [2]. In N18TG-2 neuroblastoma cells, HgCl_2 results in a significant decrease in total GSH contents [10]. Depletion of intracellular thiols through binding with mercury can alter the nature and activity of proteins within cells, possibly contributing to oxidative stress [4–6]. Therefore, providing extracellular thiol compounds, such as reduced glutathione (GSH) and *N*-acetylcysteine (NAC) can provide protection against toxic compounds that bind or oxidize (–SH) groups [28].

An important function of GSH is the protection against oxidative damage caused by ROS which are generated during normal metabolism [3]. The recycling of GSH constituents maintains homeostasis of GSH within the brain [7]. GSH is present in neurons (<1 mM) where high levels of GSH are found in astrocytes (1–20 mM) [7]. GSSG, oxidized GSH, is recycled by GSH reductase back to GSH [17].

N-acetylcysteine (NAC) enhances the synthesis of GSH and works by its direct oxygen radical scavenging effects and it potentiates the antioxidant capacity of the cells [32]. NAC has been shown to interact directly with oxidants and to protect against oxidant induced macromolecular damage and cytotoxicity in vitro. NAC may also exert its antioxidant effect indirectly by facilitating GSH biosynthesis and supplying GSH for GSH peroxidase-catalyzed reaction [14].

Although the binding chemistry of thiol groups and mercury is well known, the cellular mechanisms of GSH in modulation of the toxicity of Hg^{2+} in neuronal cells have not been examined. In this study the effects of GSH and NAC were investigated for their effects on intracellular GSH, relevant to cytoprotection against HgCl_2 .

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, heat-inactivated fetal bovine serum (FBS), Hank's balance salt solution (HBSS), phosphate-buffered saline (PBS) and penicillin/streptomycin were supplied by Fischer Scientific, Mediatech.

Cell Culture

Neuroblastoma cells, Neuro-2A (N-2A) cells were chosen for this study since they are originally derived from the brain of an albino mouse and exhibit true neuronal morphology, unlike commonly used immortal neuroblastoma cells that exhibit epithelial or fibroblastic characteristics [22]. N-2A cells were obtained from American Type Culture Collection

(Manassas, VA) and were cultured as described [21]. Briefly, cells were grown in DMEM with 10% FBS, 4 mM L-glutamine, penicillin/streptomycin (100 Units/0.1 mg/ml) and 0.02% sodium pyruvate. Each experiment was performed in a 96 well plate at a plating density of 0.5×10^6 cells/ml in low serum (1%) FBS in DMEM without methyl red. Experimental compounds were diluted in HBSS containing 2 mM HEPES, adjusted to a pH of 7.4.

Cell Viability Assays

Cell viability was determined using the Almar blue as described by [16] and [21]. The pretreatments of NAC, GSH and BSO were added 2 h prior to the addition of HgCl_2 ; then the plates were read 24 h following the HgCl_2 treatment. Briefly, Almar blue was prepared in sterile PBS at a working concentration of 0.5 mg/ml. The reduction of the dye by live cells was measured on a microplate fluorometer—Mod. # 7620—Cambridge Technologies Inc., (Watertown, MA, USA) with settings adjusted to 550/580, excitation/emission.

Glutathione Assay

The total, reduced and oxidized glutathione levels were measured according to the method described by [9]. GSH reductase recycling method was used and the cells were washed three times with ice-cold phosphate buffer saline (PBS; pH 7.4). The supernatant was used after cells were lysed for the determination of the intracellular GSH content. The pretreatments of NAC and GSH were added to the cells 24 h before treatment of HgCl_2 ; then the test system was incubated for an additional 24 h from the point of the HgCl_2 application. The cells were washed three times and lysed to measure intracellular GSH. An aliquot of cell extract (10 μl) with an aliquot of cell media (25 μl) was put in a 96 well plate and brought up to a total volume of 100 μl with 0.1 phosphate buffer containing 1 mM EDTA (pH 7.5). To this 100 μl containing 0.2 mM DTNB, 0.3 mM NADPH, and 1 unit of NADPH was added and the changes in absorbency was measured every 10 s at 415 nm for 12 consecutive readings on a microplate reader with a kinetic cartridge. Distilled water was used as blanks and standards were prepared from GSH and the slope of the rate of the reaction was used to calculate the GSH content.

Measurement of Total Thiols (TT)

Total thiols were measured using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB). DTNB, is a compound soluble in water and reacts with SH groups to produce 5-mercapto-2-nitrobenzoic acid and is used for the colorimetric determination of the SH groups. DTNB was prepared in HBSS + 2 mM HEPES buffer adjusted to pH 7.2–7.4 with 0.5 N NaOH and 0.5 N HCl to a 1 mM reagent solution. The final concentration of DTNB was 2 mM and the assays were performed in 96 well plates, which contain 0.5×10^6 cells/ml according to experimental protocol. The pretreatments of BSO and GSH were added to the cells 24 h before treatment of varying concentrations of HgCl_2 ; then the test system was incubated for an additional 24 h from the point of the HgCl_2 application. The cells were washed three times and lysed to measure intracellular TT. Color was allowed to develop for 15 min and an UV Microplate Spectrophotometer-Model 7600; version 5.02 (Cambridge Technologies Inc., Watertown, Mass) was used to measure an absorbance of 415 nm. To measure the intracellular analytes of interest, a freeze thaw method was used following careful washing of the extracellular material then freezing the plates and subsequently using the lysate to measure the analytes of interest.

Determination of Glutathione Disulfide

Glutathione disulfide (oxidized glutathione, GSSG) was determined by the method described above after treating the cells with 2-vinylpyridine [17].

Data Analyses

Statistical analysis was performed using Graphpad Prism (version 3.0), Graphpad Software Inc. (San Diego, CA, USA). Significance of difference between the groups was assessed using a one-way ANOVA, followed by a Tukey post hoc means comparison test.

Results

The viability data in Fig. 1 show the protective effect of GSH and NAC pretreatment at varying concentrations with subsequent treatment with 5 μ M of HgCl₂ to N-2A cells. GSH and NAC were cytoprotective in the N-2A cells up to 95, and 74% respectively, as compared to the cells treated with HgCl₂ only.

Figure 2 demonstrates that pretreatment of 10 mM of NAC and GSH alone did not affect cellular GSH and was slightly reduced from the control value of 13 μ M GSH to 12 μ M for both treatments. In contrast to these findings Fig. 3, shows that the pretreatment of 10 mM GSH added to N-2A cells with subsequent treatment with 5 μ M of HgCl₂ increased the cellular GSH from the control of 13 to 16 μ M. Additionally the pretreatment of 10 mM of NAC slightly decreased cellular GSH from 13 to 12 μ M (Fig. 3). Figure 4 illustrates that at high concentrations of extracellular NAC of 5 to 10 mM and subsequent treatment with 5 μ M of HgCl₂ stimulate neurite extensions and likely increase GSH synthesis. Figure 5 illustrates a similar response at 5 and 10 mM concentration of extracellular GSH which activates the N-2A cells to produce neurite extensions likely increasing GSH synthesis increasing cellular GSH.

The effects of BSO on the toxicity of HgCl₂ is presented in Fig. 6. The figure shows that cell viability declined to 66% compared to the control of 98% demonstrating that the intracellular glutathione system is a target for toxicity. In addition the data show that the inhibition of GSH synthesis using BSO with 10 mM GSH added to the media was associated with dose dependent decline in cell viability from 48% at 5 μ M BSO to 8% at 1 mM BSO compared to the control of 85% (Fig. 6). This demonstrates that GSH added to the media did not protect the cells when GSH synthesis was blocked by BSO.

In Fig. 7, 1–100 μ M HgCl₂ was added to the media after an additional 10 mM GSH was added to the media with subsequent evaluation for effects on the intracellular –SH contents. HgCl₂ from 1 to 100 μ M increased the cellular free –SH up to 24.6 μ M versus control value of 1.2 μ M. BSO added to the media with 10 mM GSH and varying HgCl₂ concentrations completely inhibited the formation of intracellular free –SH groups that were stimulated at 1–100 μ M concentrations of HgCl₂. The increase in cellular free –SH groups seen at these levels were likely due to the increase of GSH synthesis. To summarize, BSO blocked the increased concentration of –SH groups (Fig. 7) and BSO decreased viability in the presence of 5 μ M HgCl₂ with excess GSH (10 mM) indicating the mechanism of protection involves GSH synthesis (Fig. 6).

The effects of varying concentration of HgCl₂ on intracellular GSH were also evaluated and presented in Table 1. At concentrations of 1, 5 and 10 μ M HgCl₂ there was a decrease from 13 μ M for the GSH control to 5, 6 and 8 μ M intracellular GSH respectively. Then at concentrations of 100 μ M, 500 μ M and 1 mM HgCl₂ there was a decline to 4, 2 and 0.2 μ M intracellular GSH respectively. The results also show that the intracellular GSSG was inhibited at every concentration of HgCl₂ to below 0.5 μ M GSSG to GSH as compared to control of 2.1 μ M GSSG to GSH. The glutathione reductase was inactivated possibly by all concentrations of HgCl₂. The combined intracellular GSH and the GSSG to GSH cycling are added together to give the total GSH reported as a ratio in Table 1. The ratio of GSH/GSSG increased from 16.7:1 to 50:1 when HgCl₂ increased from 1 to 10 μ M. The ratio decreased from 4:1 to 0.5:1 when cells were exposed to 100 μ M–1 mM of HgCl₂.

Discussion

The high affinity of Hg for sulfhydryl groups plays a critical role in its toxicity [35]. Mercury has a high association constant for sulfhydryl groups. Hence, alteration of either intracellular or extracellular thiol status should affect cellular accumulation of Hg. In our study it was demonstrated that high levels of GSH and NAC added to the media protect the cell from mercury toxicity. Several studies have demonstrated that either alteration of intracellular thiol content or co-administration of thiols with Hg can have profound effects on the uptake and toxicity of Hg. [19] reported that exposure of renal cells to Hg concentrations above 10 μM produced marked increases in cell death. Providing extracellular thiol compounds such as GSH and NAC can provide protection against toxic compounds that bind or oxidize $-\text{SH}$ groups [28]. Recently it was observed by [23] that Mercury in even less concentration (75 and 300 nM) significantly increased glutathione level in monocyte cells. The results indicate that low concentrations of HgCl_2 stimulate the synthesis of GSH in the presence of extracellular GSH in the media. The predominate mechanism to protect the N-2A cells with extracellular NAC and GSH in the media may be related to GSH synthesis rather than just through extracellular binding.

NAC can increase cellular GSH levels, but first it must be deacetylated to cysteine, which then can be used for GSH synthesis [14]. The HgCl_2 may have formed a complex through direct binding with the $-\text{SH}$ group of the GSH and NAC in the media. In our study, GSH and NAC added to the media with the subsequent addition of HgCl_2 resulted in an increase in the cellular GSH levels. It is of interest to note that N-2A cells do not utilize the extracellular GSH and NAC to increase intracellular GSH unless there is an HgCl_2 toxicity insult. [20] examined the renal cellular concentration of glutathione (GSH) and the enzymes responsible for its synthesis, after the in vivo exposure to a sub-toxic dose of inorganic mercury (Hg^{2+}). Furthermore it was demonstrated by [20] that GSH levels and glutamylcysteine synthetase activity were increased after the exposure to Hg^{2+} . These results are consistent with the hypothesis that in vivo exposure to a sub-toxic dose of Hg^{2+} is also associated with induction of GSH synthesis and other key cellular enzymes. GSH and NAC in the media prevented the HgCl_2 from affecting cellular levels of GSH.

Treatment of the N-2A cells with buthionine sulfoximine (BSO) which is a selective inhibitor of γ -glutamylcysteine synthetase [26] inhibits GSH synthesis, was found to increase toxicity of HgCl_2 . Reduction of brain glutathione content by BSO enhances the toxic effects that are associated with elevated production of ROS [24]. When BSO was added with 10 mM GSH in the media, followed by varying concentrations of HgCl_2 , the increase of ($-\text{SH}$) groups was inhibited. This suggests that the increase in ($-\text{SH}$) groups observed at varying concentrations of HgCl_2 (1–100 μM) is due directly to stimulating GSH synthesis hence increasing cellular GSH in the N-2A cells.

As the concentration of HgCl_2 increased to 1, 5 and 10 μM , intracellular GSH decreased from the control of 13 to 5 μM , 6 and 8 μM respectively. Then from 10 μM to 1 mM HgCl_2 there was a steep decline most likely due to cell death. GSH was decreased at every level tested by HgCl_2 . Varying concentrations of HgCl_2 severely decreased the ability to convert GSSG to GSH at every level of HgCl_2 treatment down to 1 μM HgCl_2 .

In summary, data obtained in this study found that the loss of GSH plays a significant role in the toxic effects of HgCl_2 and decreases viability through decreasing cellular $-\text{SH}$ concentrations and GSH concentrations. Excess extracellular GSH and NAC were effective in elevating cellular GSH and were cytoprotective. GSH synthesis is stimulated with added pretreatment of GSH and NAC to the media with subsequent addition of HgCl_2 . This is in part due to a response by the N-2A cells related to the decreased cellular GSH. Perhaps finding an

effective way to deliver excess thiol to the brain or by stimulating the release of GSH from astrocytes could provide treatment for acute or chronic mercury toxicity. Neuro-2A cells are capable of having and generating a significant supplement of cellular GSH from extracellular sources. Isolated cells provide a good experimental model to study the mechanisms of the Hg-adaptive processes. These processes lead the toxic effect that can be compensated at the cellular level. The data presented point to the mechanisms by which external GSH and NAC can increase cellular level of GSH (especially neuronal cells). The data further indicate that the availability of GSH to the cells may not be sufficient to provide protection against mercury toxicity and the de novo synthesis of intracellular GSH is required to prevent the damaging effects of mercury.

Acknowledgments

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Fig. 1. The Protective effects of varying concentrations of GSH and NAC to subsequent exposure to 5 μ M of HgCl₂. The data represent the mean $\pm$ SEM, $n = 4$. *** $P < 0.001$

Fig. 2.
The effect of 24 h pretreatment of extracellular NAC and GSH alone on intracellular GSH concentration measured in N-2A cells, then incubated an additional 24 h as a control for Fig. 3. The data represent the mean $\pm$ SEM, $n = 24$, *** $P < 0.001$

Fig. 3.

The effect of pretreatment for 24 h with 10 mM GSH and NAC on intracellular concentration of GSH with subsequent addition of 5 μ M HgCl₂ in N-2A cells incubated for an additional 24 h. The data represent the mean $\pm$ SEM, $n = 24$, *** $P < 0.001$

Fig. 4.
The protective effect of varying concentrations of NAC on Neuro-2A cells treated with 5 μ M HgCl₂ for 24 h

Fig. 5.
The protective effect of varying concentrations of GSH on Neuro-2A cells treated with 5 μ M HgCl₂ for 24 h

Fig. 6.

The effect of glutathione depletion was investigated using BSO+ 10 mM GSH as a pretreatment with subsequent addition of 5 μ M HgCl₂ and then measuring toxicity in N-2A cells. The data represent the mean $\pm$ SEM, $n = 4$, *** $P < 0.001$

Fig. 7.

The effect of varying concentrations of HgCl₂ on intracellular free (-SH) groups in N-2A cells preincubated 24 h with 1 mM BSO and without BSO with excess GSH. The data represent the mean $\pm$ SEM, $n = 8$, *** $P < 0.001$

Table 1

The effects of varying concentrations of HgCl_2 on the intracellular GSH, GSSG, ratio of GSH/GSSG and the total GSH

mM of HgCl_2	0	0.001	0.005	0.01	0.1	0.5	1.0
μM GSH/50,000 cells	13 ± 0.03	$5 \pm 0.03^{***}$	$6 \pm 0.02^{***}$	$8 \pm 0.02^{***}$	$4 \pm 0.02^{***}$	$2 \pm 0.03^{***}$	$0.15 \pm 0.03^{***}$
μM GSSG/50,000 cells	2.1 ± 0.02	$0.03 \pm 0.05^{***}$	$0.0 \pm 0.03^{***}$	$0.14 \pm 0.01^{***}$	$0.29 \pm 0.04^{***}$	$0.48 \pm 0.05^{***}$	$0.26 \pm 0.07^{***}$
GSH/GSSG ratio	6.2	16.7	20	50	13.8	4.2	0.8
μM total GSH	15.1	5.3	6.0	8.14	4.29	2.48	0.41

The data represent the mean $\pm$ SEM, $n = 8$, $***P < 0.001$ compared to control