Potential Mechanisms of Inorganic Mercury Intoxication in Rat Kidney Cells

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ABSTRACT

Mercury is one of the ubiquitous toxic metals that is found in the environment and humans are exposed mostly via occupational and dietary sources. In the body, the primary site of accumulation and toxicity of inorganic mercury (Hg^{2+}) is proximal tubular cells. The purpose of this study was to identify the cellular mechanisms involved in Hg^{2+}-induced toxicity of target cells. Normal rat kidney (NRK) cells were exposed to several concentrations of Hg^{2+} for various times. Using imaging and biochemical techniques, Hg^{2+}-treated cells showed significant alterations in cytoskeletal structure, oxidative stress, and calcium availability. After Hg^{2+} exposure, NRK cells experienced actin disorganization and loss of cytoskeleton integrity. Additionally, intracellular levels of hydrogen peroxide and superoxide increased, which led to a rise in the amount of oxidative stress. Most interestingly, levels of calcium, a vital and strictly regulated second messenger, were elevated in the cytosol immediately following exposure. It is likely that intracellular Hg^{2+} leads to an increase in IP_3 levels, which induces calcium release from the endoplasmic reticulum. Overall, Hg^{2+}-treated NRK cells experience many adverse changes including cytoskeleton collapse, reactive oxygen species accumulation, and elevated cytosolic calcium levels. Although further studies are required for a complete understanding, a more definite explanation for the mechanisms of intracellular Hg^{2+} intoxication and cellular injury has been established.

METHODOLOGY

In order to elucidate the mechanisms of inorganic mercury intoxication, we performed biochemical, molecular, and imaging analyses in normal rat kidney (NRK) cells. It should be noted that mercuric ions bind to thiol groups with a strong affinity and thus, free mercuric ions do not exist in biological systems (1). It appears that proximal tubular cells are exposed primarily to Hg^{2+} as a conjugate of cysteine (Cys), i.e., Cys-*S*-Hg-*S*-Cys; therefore, this form of Hg^{2+} was used for the current study (2). NRK cells were exposed to buffer or Cys-*S*-Hg-*S*-Cys (10μM and 50μM) for two hours. We analyzed the effects of exposure to Cys-*S*-Hg-*S*-Cys on cytoskeleton stability, levels of oxidative stress, calcium homeostasis, autophagic processes, and mechanisms of cell death. It should be taken into account that exposure to 10μM and 50μM Cys-*S*-Hg-*S*-Cys yielded similar findings. The results of the 50μM exposure are shown here in order to fully demonstrate the effects of Cys-*S*-Hg-*S*-Cys on cells.

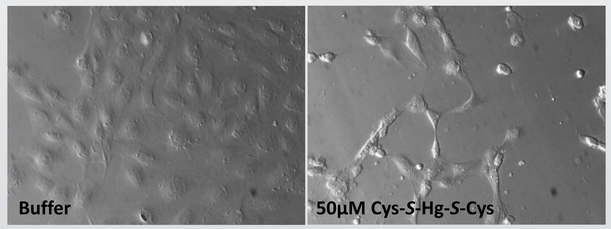

Figure 1: Photomicrographs of NRK Cells

These photomicrographs display NRK cells following exposure to various treatment groups for two hours at 37°C. The buffer-treated cells appear healthy and intact, while the Cys-*S*-Hg-*S*-Cys-treated cells are contracted and have lost their attachments to the growth surface. Cellular diameter was 53.7 ±0.9 μm for control cells and 41.6 ±1.0 μm for Cys-*S*-Hg-*S*-Cys-treated cells.

RESULTS

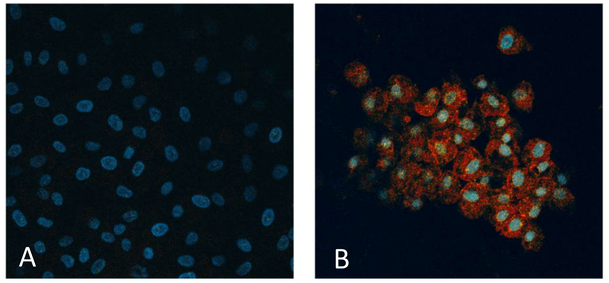

Figure 2: Mitochondrial superoxide levels

Mitochondrial oxidative stress was measured using the mitochondrial superoxide indicator, MitoSOX™ (Thermo-Fisher) and confocal microscopy. Mitochondrial oxidative stress was not obvious in control cells (A); however, in cells exposed to 50μM Cys-*S*-Hg-*S*-Cys, oxidative stress was clearly evident (B). Fluorescence intensity measured 20X greater in Cys-*S*-Hg-*S*-Cys- treated cells than control cells.

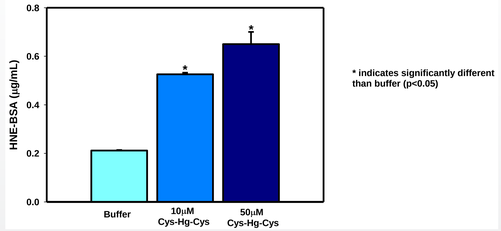

Figure 3. Levels of 4-hydroxynonenal (HNE)

4-hydroxynonenal (HNE) levels were measured in NRK cells using the HNE Adduct ELISA (Thermo-Fisher). HNE is a product of lipid peroxidation that is indicative of intracellular oxidative stress. Cells exposed to Cys-*S*-Hg-*S*-Cys had HNE levels that were significantly greater than those in control cells, suggesting that exposure to Cys-*S*-Hg-*S*-Cys induces oxidative stress.

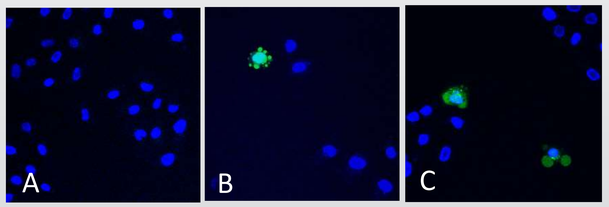

Figure 4: Role of autophagy

To examine the role of autophagy in Hg^{2+} intoxication, NRK cells were treated with Premo™ Autophagy Sensor LC3B-GFP (Thermo-Fisher) after exposure to Cys-*S*-Hg-*S*-Cys, and analyzed with confocal microscopy (Figure 4). This reagent labels LC3B, a protein essential to autophagosome formation, with green fluorescence. Control cells treated with buffer (A) exhibited almost no green fluorescence, while Cys-*S*-Hg-*S*-Cys-treated cells (B and C) had distinct spheres of green fluorescence signifying the formation of autophagosomes.

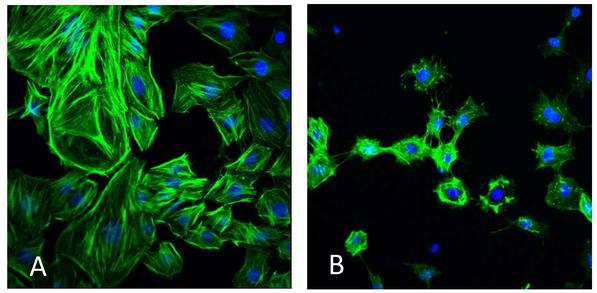

Figure 5: F-Actin rearrangement

Alexa Fluor™ 488 Phalloidin (Thermo-Fisher) is a fluorescent probe that binds to F-actin and was utilized to detect alterations in actin. Our data show that the actin cytoskeleton remains intact in control cells (A) while in Cys-*S*-Hg-*S*-Cys-treated cells, actin filaments were disorganized, cells were compressed, and the cells appeared to have lost contact with the growth surface (B). The cellular diameter was 48.9±1.2μm for control cells and 35.3±0.5μm for Cys-*S*-Hg-*S*-Cys-treated cells.

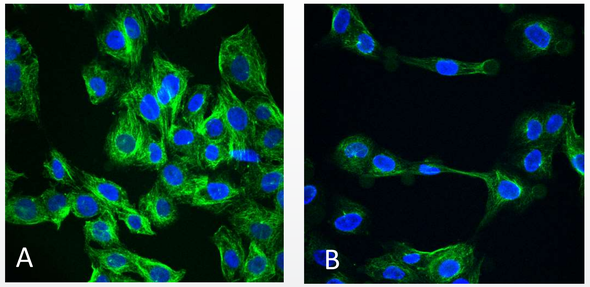

Figure 6: Microtubule rearrangement

The arrangement of tubulin proteins was studied using Tubulin Tracker Green™: Oregon Green 488 Taxol, bis-acetate (Invitrogen Molecular Probes). Tubulin Tracker indicates polymerized tubulin, or microtubules, in cells by staining with green fluorescence. Control cells (A) appear to be healthy, possess an even distribution of tubulin, and measure 51.4 ±1.3 μm in diameter. However, microtubules in Cys-*S*-Hg-*S*-Cys-treated cells (B) seemed to be fragmented or absent, which led to a reduction in cell size (43.0 ±0.7 μm).

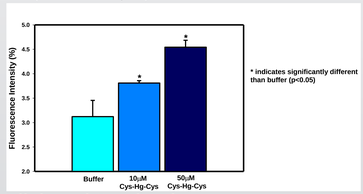

Figure 7: Intracellular calcium levels

Levels of intracellular calcium were measured in NRK cells exposed to buffer or 50μM Cys-*S*-Hg-*S*-Cys by using Fluo-3 AM Calcium Indicator (Thermo-Fisher) and flow cytometry. When cells were exposed to Cys-*S*-Hg-*S*-Cys, intracellular levels of calcium were found to be 1.5 times greater than that in control cells. We propose that the abnormal increase in intracellular calcium levels may lead to initiation of signaling pathways, which may consequently lead to dysregulation of metabolic processes within the cells.

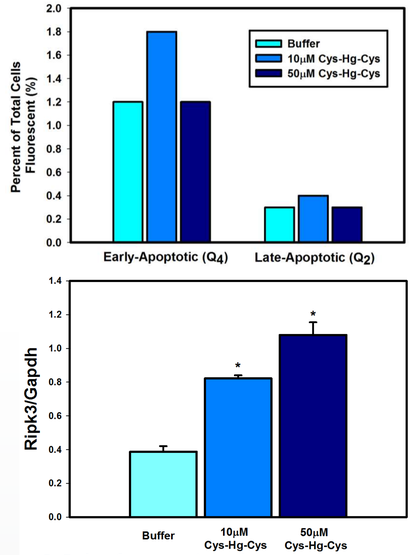

Figure 8: Role of apoptosis and necroptosis

To determine the route by which exposure to mercuric ions induces cell death, we first examined the possible role of apoptosis by measuring Annexin V using flow cytometry (Thermo-Fisher). Expression of Annexin V indicates translocated phosphatidylserine and has been shown to be a reliable marker of apoptosis. NRK cells exposed to Cys-*S*-Hg-*S*-Cys experienced slightly more apoptosis than control cells, but only 1.8% of the cells were considered apoptotic. Therefore, these data suggest that apoptosis is not the primary route of cell death in Hg^{2+} intoxication. We then chose to determine if necroptosis is involved in Cys-*S*-Hg-*S*-Cys-induced cell death. NRK cells were exposed to Cys-*S*-Hg-*S*-Cys and total RNA was isolated. Real-time PCR was then performed to determine the expression of Ripk3, a crucial protein in the necroptosis pathway. The expression of Ripk3 in Cys-*S*-Hg-*S*-Cys-treated cells was 2.8 times greater than that of control cells. These data suggest that necroptosis is a major consequence of Cys-*S*-Hg-*S*-Cys-induced cellular intoxication.

CONCLUSIONS

In summary, our data suggest that exposure of renal tubular cells to Cys-*S*-Hg-*S*-Cys leads to significant intracellular alterations in the cytoskeleton, induction of oxidative stress, altered calcium homeostasis, enhanced autophagy within cells, and ultimately, necroptosis. To our knowledge, this study represents the first time that the specific intracellular effects of exposure to Cys-*S*-Hg-*S*-Cys have been reported. Furthermore, this study is also the first to report that exposure to Cys-*S*-Hg-*S*-Cys leads to necroptosis. In general, the findings from this study provide important information regarding the specific intracellular mechanisms that lead to mercury-induced cellular intoxication and will make a significant contribution to the field of mercury toxicology.

ACKNOWLEDGEMENTS

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1) Hughes WL. A physicochemical rationale for the biological activity of mercury and its compounds. Ann N Y Acad Sci. 1957; 65(5):454-460

2) Bridges CC, Zalups RK. Molecular and ionic mimicry and the transport of toxic metals. Toxicology and Applied Pharmacology. 2005; 204:274-308.