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Mercury Alters B-Cell Protein Phosphorylation Profiles

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Abstract

Environmental exposure to mercury is suggested to contribute to human immune dysfunction. To shed light on the mechanism we identified changes in the phosphoproteomic profile of the WEHI-231 B cell line after intoxication with Hg²⁺. These changes were compared to changes in the phosphoproteome that were induced by pervanadate or okadaic acid exposure. Both 250 μM HgCl₂ and pervanadate, a known phosphotyrosine phosphatase inhibitor, caused an increase in the number of proteins identified after TiO₂ affinity selection and LC-MS/MS analysis. Pervanadate treatment had a larger effect than Hg²⁺ on the number of Scansite motifs which were tyrosine-phosphorylated, 17, and Ingenuity canonical signaling pathways activated, 4 with score > 5.0. However, Hg²⁺ had a more focused effect, primarily causing tyrosine-phosphorylation in SH2 domains in proteins that are in the B cell receptor signaling pathway. The finding that many of the changes induced by Hg²⁺ overlap with those of pervanadate, indicates that at high concentrations Hg²⁺ inhibits protein tyrosine phosphatases.

Keywords

mercury; Hg²⁺; toxicology; B cell; WEHI-231; phosphoproteomics; phosphoprotein; phosphotyrosine; TiO₂; mass spectrometry

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Author Contributions

The manuscript was written through contributions of all authors. Nicholas J. Carruthers: data analysis, interpretation of findings and manuscript preparation. Paul M. Stemmer: study design, sample preparation, data analysis, interpretation of findings and manuscript preparation. Namhee Shin: sample preparation and mass spectrometry. Alan Dombkowski: data analysis. Joseph A. Caruso: mass spectrometry and data analysis. Randal Gill: sample preparation. Allen Rosenspire: study design, interpretation of findings and manuscript preparation. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

All proteins identified along with their protein probabilities and the number of peptides assigned to that protein are listed in supplementary tables S1: "Proteins Identified in the 250 μM HgCl₂/okadaic acid experiment", and S2: "Proteins Identified in the 100 μM HgCl₂/pervanadate experiment". For all proteins with only one peptide assignment the information regarding the quality of the identifications is listed in supplementary tables S3: "Peptide identification data for proteins found in the 250 μM Hg²⁺/okadaic acid experiment by only one peptide" and S4: "Peptide identification data for proteins found in the 100 μM HgCl₂/pervanadate experiment by only one peptide". This material is available free of charge via the Internet at <http://pubs.acs.org>. The mass spectrometry data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository⁵³ with the dataset identifier PXD000312 and DOI 10.6019/PXD000312.

INTRODUCTION

Mercury is distributed in the global environment by both natural geologic processes and human activities¹². Consequently large segments of the world's population are exposed to the metal through water, air, food, and dental amalgam. Mercury is clearly toxic, and exposure to high or even moderate levels of organic methyl mercury (MeHg), inorganic mercury (Hg^{2+}) or metallic mercury (Hg^0) is well established to damage the nervous and immune systems, resulting in neurological and immunological deficiencies in humans³⁻⁵. Epidemiological studies now suggest that exposure to environmental mercury at current common levels, which have been perceived to be non-toxic, may contribute to immune system dysfunction and development or progression of autoimmune disease in humans⁶⁻¹². This view is supported by experiments in mouse models of autoimmune disease. In most genetically autoimmune prone mouse strains, exposure to low levels of Hg^{2+} exacerbates disease¹³⁻¹⁶, and in mice not prone to autoimmunity low levels of Hg^{2+} exacerbate disease in several models of induced (acquired) autoimmunity^{6,17,18}.

Autoimmune disease arises when autoreactive B and T cells, which are normally held in check by mechanisms collectively known as central and peripheral tolerance, become de-suppressed. Central tolerance refers to the mechanisms by which newly developing immature T cells and B cells are rendered non-reactive to self. These mechanisms are distinct from peripheral tolerance in that they involve the T Cell Receptor (TCR) and B Cell Receptor (BCR) clonotypic receptors and occur only in immature cells, prior to export into the periphery. Peripheral tolerance requires activation of other receptor systems and develops only after T and B cells mature and enter the periphery. Disruption of either mechanism can result in autoimmune disease. Unfortunately most of the studies connecting mercury to autoimmune disease give little guidance as to what the mechanism or mechanisms responsible for the linkage might be. In other words, we do not know how exposure to mercury compromises central and/or peripheral tolerance. This lack of information represents a major gap in our understanding of how environmental mercury interacts with the immune system to promote the development of autoimmune disease.

Recently there has been increased recognition of the essential role played by auto-reactive B cells and of autoantibodies produced by B cell derived plasma cells in the pathology of a variety of autoimmune diseases¹⁹. To a large extent B cell (as well as T cell) tolerance depends upon phosphoprotein mediated signaling. Since mercury inhibits cellular phosphatase activity²⁰, one possible mechanism for mercury disruption of central tolerance in B cells is the inhibition of critical phosphatases resulting in an aberrant increase in protein phosphorylation. In the studies described here, we have utilized modern mass spectroscopic technology to investigate the effect of mercury on the phosphoproteome in the WEHI-231 mouse B cell line, a well-studied model system known to possess many of the characteristics of immature B cells. The objectives of this study were to determine whether mercury caused an increase in protein phosphorylation in B cells and to identify specific phosphoproteins and their associated signaling pathways most likely to be molecular targets of mercury.

Global phosphoproteomic analysis has recently become possible through the application of affinity selection of phosphopeptides and mass spectrometric identification of those

peptides²¹⁻²³. Specifically, the WEHI-231 cell line has been profiled resulting in identification of 107 phosphoproteins and 193 phosphorylation sites in one study²⁴ and over 400 proteins in another²⁵. This is a relatively modest slice of the entire phosphoproteome as it is predicted that up to 100,000 sites could be phosphorylated in the complete proteome²⁶. Of equal or even greater importance in the profiling of phosphopeptides is the quantification of the change in phosphorylation after an experimental intervention or environmental exposure. Mass spectrometry enables multiplex analysis by using isotopically distinct labels for different samples^{27,28} but can also be used in a label independent way by quantitating based on the number of spectra observed^{29,30}. For instance a recent study utilizing these methods examining global protein phosphorylation dynamics during deoxyvynivalenol-induced ribotoxic stress in macrophages has identified extensive and unexpected changes to the macrophage phosphoproteome³¹. For the work presented in this paper we quantified the abundance of phosphopeptides and phosphoproteins using the spectral counting strategy.

Material and Methods

Materials

Acetic Acid was from BDH Chemicals (through VWR, Radnor, PA), CaCl₂ was from Orion, TFA (trifluoroacetic acid) was from Fisher Scientific (Pittsburgh, PA) and formic acid was from EMD. (Billerica, MA). TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated bovine pancreas trypsin and all other reagents including solvents used for HPLC were the highest grade available from Sigma-Aldrich. (St Louis, MO). TiO₂, 5 micron, was from GL Sciences.

Cell Culture

WEHI-231 cells were obtained from the American Type Culture Collection. Cells were maintained on RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μM mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO₂ atmosphere. Cells were passaged three times a week and harvested for experiments while in logarithmic growth.

Exposure and Harvesting

Cells were pelleted then resuspended at 5×10^6 cells per ml for exposure to HgCl₂ (Hg²⁺) at 100 μM or 250 μM, pervanadate at 25 μM or okadaic acid at 100 nM. Following a 10 minute exposure in RPMI 1640 to the test agent the cells were washed twice in ice cold Hanks' solution then pelleted and frozen. The treatments were divided into two separate experiments: the first experiment included 250 μM Hg²⁺, 100 nM okadaic acid and an untreated control, the second experiment included 100 μM Hg²⁺, 25 μM pervanadate and an untreated control. The first experiment was carried out using 3 independent samples per treatment and the second experiment with 4 independent samples per treatment.

Sample Preparation

Cell pellets were lysed in Tris, 20 mM pH 8.0, SDS, 0.5%, LiFl, 1.0 mM and Na₃VO₄, 0.1 mM then supernatant from a 20,000 × g centrifugation was taken for analysis. Protein in lysates was determined using a BCA assay (bicinchoninic acid assay; Pierce, Rockford, IL).

Samples were digested overnight with 1:100 trypsin at a sample protein concentration of 1.0 mg/ml in buffer containing Tris, 20 mM, SDS, 0.1%, LiF, 1.0 mM, Na₃VO₄, 0.1 mM and 10% acetonitrile. All samples were evaluated by SDS-PAGE to ensure full digestion of the samples before proceeding to phosphopeptide isolation. Phosphopeptides were isolated from 1 mg of digested cell protein using 2 sequential incubations each with 2 mg of 5 μ TiO₂ beads for the first experiment.

Phosphopeptide enrichment for the first and second affinity selections was 89 and 93 %, respectively, indicating that additional phosphopeptides could be harvested by increasing the ratio of TiO₂ beads to protein without compromising the selectivity. Therefore, the second experiment used a single incubation with 6 mg of 5 μ TiO₂ beads for each sample. The selectivity of the 6 mg of 5 μ TiO₂ procedure was 98%. Cell digests were incubated with TiO₂ in 2% TFA saturated with glutamic acid in 60% acetonitrile. The beads were washed three times with 1% TFA in 60% acetonitrile before eluting phosphopeptides with NH₄OH in 50% acetonitrile. TiO₂ eluates were neutralized with formic acid, dried under vacuum and stored at -80°C until analysis. Eluted peptides solubilized in 0.1% formic acid were then analyzed by LC-MS/MS without further purification.

Mass Spectrometry

All analyses were performed on a Thermo LTQ equipped with ETD (ThermoFisher Scientific, Watham, MA). Samples were loaded on a peptide Captrap (Michrom, Auburn, CA) trapping column and peptide separations achieved using a linear gradient of 5% to 35% acetonitrile to elute from a Majic 0.1 mm $\times$ 150 mm AQ C18 column (Michrom). The first experiment had data from two TiO₂ selection steps that were run separately on the mass spec. The .raw files were combined during database searching and a total of 233,195 spectra from 9 samples were submitted for that experiment. The second experiment, with 100 μ M Hg²⁺ or pervanadate, has only one TiO₂ elution. Those 12 samples yielded a total of 239,443 spectra. LC-MS/MS for the second treatment group was run in a neutral loss mode so that high abundance precursor neutral losses of 24.25, 32.66, or 49.00 m/z found in an MS2 spectrum were selected for MS3 analysis. The first experiment LC-MS/MS did not incorporate a neutral loss scan.

Database Searching

Tandem mass spectra were extracted by Proteome Discoverer (ThermoFisher Scientific) version 1.4.0.288. Charge state deconvolution and deisotoping were not performed. All MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot and X! Tandem were each set up to search the 2013_04 release of the SwissProt database (selected for *Mus musculus*, 16611 entries) assuming the digestion enzyme trypsin. Spectra were searched with a fragment ion mass tolerance of 0.70 Da and a parent ion tolerance of 3.5 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine, acetylation of the n-terminus and phosphorylation of serine, threonine and tyrosine were specified as variable modifications.

Criteria for Protein Identification

Scaffold (version 4.0.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm³². Protein identifications were accepted if they contained at least one identified phosphopeptide and had a Protein Prophet probability greater than 80%. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. All subsequent analysis of protein sets included all proteins or peptides that met the criteria for identification without weighting for the level of confidence in the identification.

Ascore Filtering

The Ascore algorithm was applied to phosphopeptides that met identification criteria and had multiple possible phosphorylation sites in order to determine which site or sites were likely to be modified and the confidence in those localizations³³. Site localization was accepted if the Ascore algorithm indicated at least 95% confidence in the assignment. Peptide identification data were imported into Scaffold PTM (Version 2.1.1, Proteome Software) software where the Ascore algorithm was implemented.

Motif Analysis

Ascore-filtered phosphorylation sites were searched against a database of putative protein kinase target motifs and phosphorylation-dependent binding motifs predicted by Scansite³⁴. Scansite uses data from oriented peptide library and phage-display experiments to generate position-specific scoring matrices that are queried against sequence databases to predict kinase target and domain binding sites. Our data were searched against the high stringency Scansite data that only include sites that fell within the top 0.2% of scores for each motif. All identified proteins were submitted to the Scansite web service (<http://scansite3.mit.edu>). Motifs identified in those proteins were retrieved and the invariant residue (the modified residue) for each was searched against our Ascore-filtered phosphorylation sites

Pathway Enrichment Analysis

Sets of proteins that were modified by the treatments were examined for overrepresented pathways using the IPA (Ingenuity Systems Inc., Redwood City, CA) Global Canonical Pathway Analysis (Content version: 14855783 (Release Date: 2013-02-05)). Pathways in the ingenuity canonical pathway database were tested for significant associations with the protein sets described in the results section using Fisher's exact test with all mouse genes in the ingenuity knowledgebase as background.

Statistical Analysis

Statistical tests were carried out in R version 3.0.0 except for the ANOVA tests of protein normalized spectral counts which were carried out in DanteR version 0.2³⁵. The sets of modified proteins were maximized for subsequent analysis by accepting statistical test results without correction for multiple testing unless otherwise stated. Proportional Venn diagrams were made with the EulerAPE tool available at <http://www.eulerdiagrams.org/>

eulerAPE/. Where a value and range are reported, those values represent the mean and standard deviation.

RESULTS

The depth of phosphoproteome coverage and the magnitude of global phosphorylation changes observed in WEHI-231 cells treated with Hg^{2+} , okadaic acid or pervanadate are shown in figure 1. Two separate experiments were run to evaluate the ability of Hg^{2+} to modulate the phosphoproteome in WEHI-231 cells. The TiO_2 selection was effective for phosphopeptides, as indicated by 92% and 98% of all identified peptides being phosphopeptides in the first and second experiment, respectively. In the first experiment, (Figure 1, panels A & B; Supplemental Tables S1 and S3) WEHI-231 cells were exposed to 250 μM Hg^{2+} or 100 nM okadaic acid for 10 minutes. The concentrations of each agent were selected to achieve a maximal effect on Hg^{2+} -responsive pathways and phosphatase Type-1 and Type-2A substrates, respectively. In this experiment 688 proteins were identified with 95% confidence at the peptide level and a 0.7% false discovery rate at the protein level. The names of proteins in the groups depicted in figure 1 A and B are provided in supplementary tables S1 and S2 respectively. In addition, for proteins that were identified by only one peptide, the Peptide Prophet scores and mass deviations are provided in supplementary tables S3 and S4. All protein identifications and mass spectra have been submitted to the PRIDE database.

In an attempt to collect additional phosphopeptides and achieve greater depth of coverage the second experiment used 6 mg of TiO_2 per mg of protein for the phosphopeptide selection, rather than the 4 mg used in the first experiment. Changing the experimental procedure for TiO_2 selection of phosphopeptides had no discernible effect on the number of proteins or peptides identified in any run, indicating that a representative set of phosphopeptides was selected each time. The addition of a neutral loss scan in the second experiment resulted in a 4.3 % decrease in the number of phosphosites identified in control samples with no change in the distribution of spectra based on the type of phosphorylated residue. This indicates that for the TiO_2 selected samples in this study the depth of coverage was limited by the degree of chromatographic fractionation after phosphopeptide selection.

A review of the literature has shown that mercury can interact with cellular systems in a great variety of ways³⁶. It is also known that protein phosphatases, and protein tyrosine phosphatases in particular, are susceptible to oxidation because they contain cysteine residues in their active sites³⁷. As mercury is well known to irreversibly bind to cysteine residues³⁸, we hypothesized that one prominent mechanism by which mercury could modulate the phosphoproteome would be via tyrosine phosphatase inhibition. In addition, several phosphoserine/phosphothreonine phosphatases are sensitive to oxidative stress³⁹. Mercury could act to inhibit phosphatase activity by direct sulfhydryl binding in active site Cys residues or indirectly via oxidative stress.

An increase in cellular protein phosphorylation should result in an increase in the number of phosphoproteins and phosphopeptides detected by mass spectrometry after TiO_2 phosphopeptide selection. When the data for Hg^{2+} exposed cells were compared to control,

the 250 μM Hg^{2+} caused the number of phosphoproteins and unique phosphopeptides to increase by 166 and 321. Normalized spectral counts were used to measure protein abundance and ANOVA to identify proteins that had altered abundance in response to treatment. This analysis identified 180 proteins that had changes ($p < 0.05$) in spectral count abundance. Treatment with 250 μM Hg^{2+} , in addition to increasing the number of peptides and proteins identified, also increased the variability in the proteins that were identified between replicate runs so that 30.0% of the proteins identified in 250 μM Hg^{2+} treated cells were observed in just one of the three samples while only 21.4% were identified in just one control sample and 24.0% of proteins were identified in just one of the okadaic acid treated samples. Proteomics experiments using data dependent analysis typically identify variable sets of proteins between technical replicates so that 30% or more of proteins in one analysis won't be found in a replicate analysis⁴⁰. The data presented here were from independent biological samples that are expected to have greater variability than technical replicates so the estimate of 30% variability between samples represents a reasonable best case outcome.

In the second experiment (Figure 1, panels C & D; Supplemental Tables S2 and S4), the Hg^{2+} concentration was decreased to 100 μM to achieve greater specificity of action and pervanadate, a known inhibitor of phosphotyrosine phosphatases, was introduced as a positive control. Pervanadate acts, as we hypothesize mercury acts, by two mechanisms for inhibition of pTyr phosphatase activity: directly by competitive phosphatase inhibition or indirectly by oxidative inactivation⁴¹. It was used at 25 μM to achieve maximal inhibition of phosphotyrosine phosphatases. In the second experiment 607 proteins were identified with 95% confidence at the peptide level and a 1.8% false discovery rate at the protein level. Pervanadate treatment increased the number of phosphoproteins by 51 and phosphopeptides by 91 over control. ANOVA identified 160 proteins that had changes in normalized spectral counts ($p < .05$) due to pervanadate treatment and 53 that had changes due to 100 μM Hg^{2+} . Like Hg^{2+} , pervanadate increased the variability of protein identifications between samples, resulting in 24.7% of all identified proteins being identified in just one out of four samples, compared to 18.9% in controls. Cells treated with 100 μM Hg^{2+} had 22.4% of their proteins identified in just one out of four samples.

In the two experiments the 250 μM Hg^{2+} and the 25 μM pervanadate groups have the greatest number of phosphoproteins and phosphopeptides identified. In contrast, the actions of 100 nM okadaic acid and 100 μM Hg^{2+} were very modest. Okadaic acid caused an increase of only 56 proteins and 76 peptides over control and the 100 μM Hg^{2+} exposure group had a decrease of 8 proteins and 4 peptides compared to control (table 1). Protein spectral counts were compared between okadaic acid treated and control cells using ANOVA and 37 proteins were found to be different at the $p < 0.05$ level. Those proteins are highlighted in gray in supplementary table S1. Table 1 shows the number of proteins and the number of peptides that change for each treatment group as well as the number that are significantly different from control or that change by 2 fold or greater in spectral counts.

The Ascore algorithm³³ was used to localize phosphorylation sites with high-confidence. For the site assignment analysis, peptides were not assembled into proteins and no attempt was made to find the minimal list of proteins required to explain all matched spectra. If a sequence appeared in more than one protein then all possible protein assignments were

included in the set of identified proteins for subsequent analysis. In the 250 μM Hg^{2+} /okadaic acid experiment, 2136 possible phosphorylation sites were submitted for Ascore analysis which determined that 1388 of them, 65.0%, had site localization evidence that surpassed 95% confidence. In the 100 μM Hg^{2+} /pervanadate experiment, 891 of 1641 possible phosphorylation sites, 54.3%, had greater than 95% site localization confidence. Data were aggregated by treatment to determine the assignment of pSer, pThr and pTyr residues. Over 90% of phosphopeptide sites identified were pSer in the control samples and the ratio of pSer : pThr phosphopeptides was constant at 12 : 1 for all conditions evaluated. The number of pTyr phosphopeptides identified was increased by the exposure to 100 μM or 250 μM Hg^{2+} and most dramatically by exposure to 25 μM pervanadate, (figure 2). The number of pTyr peptides identified increased from an average of 5.3 ± 1.7 in controls to 20.3 ± 1.3 and 95.8 ± 20.8 in the 100 μM Hg^{2+} or pervanadate treatment groups, respectively. In the first experiment the number of pTyr peptides identified increased from 4.0 in control to 33.7 ± 3.1 and 1.3 ± 0.6 in the 250 μM Hg^{2+} or okadaic acid treatment groups, respectively. It is notable that although the exposures to Hg^{2+} increased the number of different phosphoproteins and phosphopeptides identified without changing the number of different pSer sites identified using the Ascore algorithm (figure 2), the number of spectral counts assignable to pSer residues in the 250 μM Hg^{2+} group is smaller than in the control group (1916 ± 59 in 250 μM Hg^{2+} vs 2570 ± 64 in controls, t-test p-value=0.0018).

Two approaches were used in evaluating the proteins involved in the cellular response to Hg^{2+} : motif analysis using Scansite³⁴ and pathway analysis using IPA. For the motif analysis, the Ascore-filtered sites were searched against the Scansite database and then the numbers of sites that matched each Scansite motif were counted. For presentation, the data were split into pSer/pThr sites (figure 3) and pTyr sites (figure 4). In the 250 μM Hg^{2+} /okadaic acid experiment, 332 of 1461 (23%) sites searched had identified motifs (figure 3 A and 4 A). In the 100 μM Hg /pervanadate experiment, 226 of 943 (24%) had motif assignments (figure 3 B and 4 B). Scansite has high specificity and low sensitivity relative to other phosphosite prediction algorithms and assignment of kinase motifs for less than 50% of the submitted sites is reasonable⁴².

In the combined data set from all treatments, the motifs for 10 SH2 binding domains and 7 kinases were discovered. The pTyr motifs that were found in cells treated with 250 μM Hg^{2+} were mainly binding sites for SH2 domain proteins. This is consistent with the predominance of SH2 domains in phosphotyrosine signaling⁴³. Nine sites were in SH2 motifs including Y182 and Y193 of CD79A and Y195 and Y206 of CD79B, which constitute the ITAM domains of those proteins, Y935 of PTPRC (Receptor-type tyrosine-protein phosphatase C) and Y342 of KSYK (Tyrosine-protein kinase SYK). Several of those sites, including Y182 and Y193 of CD79A, Y935 of PTPRC and Y342 of KSYK, were also found among the SH2 motifs in 100 μM Hg^{2+} treated cells.

Phosphoproteins that had different spectral count abundance as determined by ANOVA, $p < 0.05$, were submitted for canonical pathway analysis in Ingenuity. The top scoring, non-redundant pathways for Hg^{2+} and pervanadate treatments are shown in table 2. The B cell receptor signaling pathway was the top hit for 100 μM Hg^{2+} and pervanadate treated cells and the second hit for 250 μM Hg^{2+} treated cells. The eIF2 signaling pathway was the top

hit for 250 μM Hg^{2+} treated cells. Phosphoproteins with abundance changes induced by okadaic acid treatment had lower scores for pathway enrichment than the other treatments: eIF2 signaling was its second highest hit.

Pathway and cellular process enrichment for proteins that contained phosphotyrosine residues was evaluated by submitting proteins with Ascore-filtered Tyr phosphosites that had at least 3 spectral counts in any replicate to Ingenuity canonical pathway analysis, (table 3). Ten proteins met the submission criteria for 250 μM Hg^{2+} treated cells, 4 for 100 μM Hg^{2+} treated cells and 32 for pervanadate treated cells. The four pTyr proteins from the 100 μM Hg^{2+} group that met the selection criteria are all associated with the B-cell receptor signaling pathway. That pathway is also the top scoring pathway for the 250 μM Hg^{2+} group where it is separated from the next highest scoring pathway by almost 7 order of magnitude change in p-value. Pervanadate treated cells had tyrosine-phosphorylated proteins in several other pathways including 14-3-3-mediated signaling, remodeling of epithelial adherens junctions and axonal guidance. Figure 5 is an adaption of the KEGG map⁴⁴ for the B-cell receptor signaling pathway that indicates the level of tyrosine phosphorylation for identified proteins. All of the proteins in the KEGG pathway map that were identified as phosphoproteins are included irrespective of the findings from the Ingenuity analysis.

Phosphotyrosine sites identified in the Hg^{2+} treated cells were concentrated in just a few proteins. Of the average 90 Ascore-filtered spectral counts spread over 38 proteins in the 250 μM Hg^{2+} treated cells, $54\% \pm 8.7$ of these were in the two protein, PTN6 and CD79A. Similarly, $57\% \pm 3.9$ of pTyr spectral counts in 100 μM Hg^{2+} treated cells were found in those same two proteins. In pervanadate treated cells, the proteins PTN6 and CD79A accounted for an average of only $12\% \pm 0.9$ of pTyr spectral counts.

Figure 6 shows pTyr spectral counts at sites in selected proteins in the B-cell signaling pathway. Phosphorylated CD79B total spectral counts were greatly increased by Hg^{2+} treatment (figure 5), and all spectral counts for that protein came from the peptide spanning A187 to R213_which contains its ITAM signaling domain. The Ascore filter removed 98.0% of its possible site-based spectral counts so this protein had a low signal in Ascore filtered data and is not included in this figure.

Discussion

We used a ten minute exposure to high doses of Hg^{2+} to explore the hypothesis that it would act as an inhibitor of phosphatases and so increase protein phosphorylation in the WEHI-231 B cell model. Exposure to 250 μM Hg^{2+} had a profound effect on the phosphoproteome of WEHI-231 cells. The numbers of phosphoproteins and phosphopeptides were increased in comparison to controls (figure 1, table 1) as was the number of pTyr sites identified (figure 2) and the abundance of phosphoprotein components of the B cell receptor signaling pathway were dramatically increased (figures 5 and 6). This pathway is intimately involved in regulating B cell tolerance, the mechanism by which the B cell repertoire is pared of self-reactive B cells⁴⁵. Not surprisingly, disruption of the B cell receptor pathway has been shown to be linked to autoimmune disease^{46,47}. We have presented evidence that Hg^{2+} can increase protein phosphorylation in a B cell model, particularly in components of the B cell

receptor signaling pathway, suggesting this as a possible mechanism for disruption of central tolerance by mercury.

Neutral loss scans were included in the second experiment in an attempt to increase the depth of coverage for phosphoproteins. For the entire data set for experiment two, when the neutral loss scans are included, 239,443 spectra were submitted resulting in 607 phosphoproteins and 891 phosphosites identified. Without the neutral loss scans 187,716 spectra were submitted and 581 proteins and 853 phosphosites were identified. The modest increase in identified phosphoproteins and phosphosites when neutral loss spectra were included shows that the addition of those spectra were not a major factor in expanding the depth of coverage for the phosphoproteome. The possibility that the additional time required for acquiring neutral loss spectra in the experiment would decrease the depth of coverage of all phosphoproteins or of phosphopeptides with a specific type of residue, such as pTry peptides, can not be completely discounted. This possibility is supported by data for the total number of phosphoproteins and phosphopeptides identified in the untreated samples. In the two experiments 343 ± 13 and 306 ± 16 phosphoproteins and 535 ± 28 and 428 ± 24 phosphopeptides were identified in the experiments without and those with neutral loss scans, respectively. Adding the neutral loss scan did not change the ratio of pSer/pThr to pTry phosphopeptides that were identified, pTyr represented 3.1 ± 0.49 % of all phosphorylated residues assigned by the ASCORE algorithm when the neutral loss scan was included and 3.4 ± 0.31 % when those scans were not submitted for analysis. The pervanadate and the 100 μ M Hg treatment groups were both in the experiment that incorporated neutral loss scans and they have the highest and second lowest number of pTyr sites identified. Taken together these data indicate that including the neutral loss scans in the experiment did not have a major effect on either the depth of phosphoproteome coverage or on the type of phosphosite that was detected. The limiting factor for the depth of coverage for this analysis was, therefore, the degree of fractionation after the phosphopeptide selection step. Since, in all instances, this was a single 2 hour gradient on a reversed phase column, the results from the two experiments are comparable.

Exposure to Hg^{2+} at the lower concentration of 100 μ M caused a slight decrease in the numbers of phosphoproteins and phosphopeptides identified in comparison to untreated cells (table 1). The 100 μ M Hg exposure group did have an increase in pTyr sites identified (figure 2) and an increase in the abundance of phosphoproteins and pTyr sites in the B cell signaling pathway (figures 5 and 6, table 3). This confirms that increased pTyr signaling in the B cell receptor pathway is a core effect of Hg^{2+} in WEHI-231 cells. Although Hg^{2+} would be expected to bind to most protein cysteine residues after exposure to either the 100 or 250 μ M concentrations used for these studies, Hg^{2+} is positively charged and it has been shown that the ability of Hg^{2+} to cross the plasma membrane is a function of concentration. Because of this barrier, at lower exposure levels the cytoplasmic Hg^{2+} availability is more limited⁴⁸. It is possible that the effects of Hg^{2+} on the phosphoproteome which we find after 100 μ M exposures are limited to the consequences of Hg^{2+} binding to external cysteine, while the differences observed after exposure to 250 μ M Hg^{2+} reflect changes due to Hg^{2+} binding to both the external and the cytoplasmic cysteines.

In order to establish the effects of exposing cells to known phosphatase inhibitors we treated WEHI-231 cells with okadaic acid and pervanadate. Okadaic acid is the prototypical phosphatase inhibitor that has high specificity for the major pSer/pThr phosphatases whereas pervanadate inhibits pTyr phosphatases. The effect of okadaic acid on the WEHI-231 phosphoproteome was modest in comparison to the other treatments used (figure 1) and only the AuroB phosphorylation motif was increased in representation in the data by okadaic acid. Although 100 nM okadaic acid is typically a maximally effective concentration for inhibiting both Type-1 and Type-2A pSer/pThr phosphatases, in our experiment the full effect of that inhibition may not have been observed because the duration of exposure may have been insufficient to allow a general increase in the phosphorylation state of cellular proteins in the unstimulated cell system we used for these studies. Hg^{2+} and pervanadate are less specific and act on a broader range of substrates as well as subjecting the cells to an oxidative environment that would cause an activation of signaling pathways in the cells as they respond to the oxidative stimulus. Pervanadate, like 250 μM Hg^{2+} stimulated a robust response in the WEHI-231 cells. The numbers of phosphopeptides, phosphoproteins and pTyr sites were all increased in comparison to untreated cells (figures 1 and 2 and table 1). The abundance of phosphoproteins and the number of pTyr sites in the B cell signaling pathway were both increased (figures 5 and 6, tables 2 and 3). Overall, the phosphoproteomes of 250 μM Hg^{2+} treated cells and pervanadate treated cells are similar to each other, consistent with the hypothesis that they share the same mechanism of action. This is consistent with Hg^{2+} inhibiting pTyr phosphatases through inactivation of cysteines in the active sites of pTyr phosphatases as is well established for pervanadate, but the action of Hg on the phosphoproteome may not be limited to this one mechanism. However the two treatments differ from each other in two ways. First, 250 μM Hg^{2+} had some effect on Ser/Thr signaling as indicated by the enrichment of 9 pSer/pThr motifs in 250 μM Hg^{2+} treated cells compared to untreated while pervanadate treatment had a smaller effect with the enrichment of only 4 pSer/pThr motifs (figure 3). Second, pervanadate stimulated the phosphorylation of a broader range of Tyr residues than did the Hg^{2+} treatment. This is indicated by the greater number of pTyr residues identified in pervanadate treated cells in comparison to 250 μM Hg^{2+} treated cells (95.8 vs. 33.7 sites, figure 2) and the greater number of pTyr motifs that were enriched in pervanadate treated cells in comparison to Hg^{2+} treated cells (10 motifs compared to 6 motifs, figure 4). In addition, as noted above, over 50% of the Ascore filtered pTyr spectral counts in cells treated with either concentration of Hg^{2+} were accounted for by just two proteins in the B cell receptor signaling pathway, PTN6 and CD79A, while those proteins accounted for only 12% of pTyr spectral counts in pervanadate treated cells. These data suggest that Hg^{2+} has a more narrow range of targets in pTyr stimulation in comparison to pervanadate.

Up to this point our findings are consistent with the view that pervanadate and Hg^{2+} each modulate Tyr phosphorylation by inhibiting phosphotyrosine phosphatases. The mechanism for that inhibition is likely to be redox dependent inactivation of cysteines that are present in the active site of the protein tyrosine phosphatases. In fact, earlier work based on anti-pTyr western blots found that exposure of lymphocytes to similar concentrations of Hg^{2+} as were used here also resulted in extensive redox-linked ligand-independent increases in protein tyrosine phosphorylation^{50,51}. Pervanadate was the most effective of all the agents tested in

increasing both the number of pTyr peptides identified and the number of pTyr motifs. However, both concentrations of Hg^{2+} tested also increased the number of pTyr peptides and motifs identified.

Phosphotyrosine identification in Jurkat and Primary T-cells was able to identify up to 299 proteins with phosphotyrosine⁵². However the majority of studies found between 75 and 168 phosphotyrosine sites⁵². In WEHI-231 cells 193 phosphorylation sites were identified and mapped to 107 proteins using IMAC²⁴. Given the 679 phosphoproteins and the 1168 phosphorylation sites identified in this study it is clear that these data represent a meaningful examination of the phosphoproteome and the actions of pervanadate and mercury upon it.

The massive disruption of the phosphoproteome after exposure of WEHI-231 cells to high levels of Hg^{2+} is consistent with the observation that, at high levels, Hg^{2+} is extremely toxic, leading to rapid cell death⁴. This is consistent with the finding that proteins in the eIF2 signaling pathway were more abundant in the phosphopeptides isolated from cells treated with Hg^{2+} . Phosphorylation of eIF2 prevents that protein from initiating protein synthesis and is an indicator of cell stress⁴⁹. However, at lower and more environmentally relevant levels, Hg^{2+} is not cytotoxic but, nevertheless, is linked with adverse autoimmune effects⁶⁻¹². A likely explanation for this is that at lower concentrations, Hg^{2+} acts much more selectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

pTyr	phosphotyrosine
pSer	phosphoserine
pThr	phosphothreonine
RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
BCA assay	bicinchoninic acid assay
ETD	electron-transfer dissociation

LC-MS/MS	liquid chromatography with tandem mass spectrometry
ANOVA	analysis of variance
ITAM	(immunoreceptor tyrosine-based activation motif)
TCR	T cell receptor
BCR	B cell receptor
SH2	src homology 2
KEGG	Kyoto encyclopedia of genes and genomes
IPA	Ingenuity pathways analysis

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Figure 1.

Identification of phosphoproteins and phosphopeptides isolated from WEHI-231 cell. Proteins, panels A & C, or phosphopeptides, panels B & D, identified using TiO_2 -selected phosphopeptides from untreated cells or cells treated with 250 μM Hg^{2+} or 100 nM okadaic acid (A & B), or 25 μM pervanadate or 100 μM Hg^{2+} (C & D). Untreated sample regions are highlighted in grey. Numbers indicate total proteins identified in the group (A & C) or the number of unique phosphopeptides identified in the group (B & D). $n = 3$ (A & B) or 4 (C & D).

Figure 2.

Hg^{2+} and pervanadate increase tyrosine phosphorylation for Ascore-filtered sites. Panel “A” shows the number of pSer (white bars), pThr (grey) and pTyr (black) sites identified in each group for the first exposure condition, $n = 3$. Panel “B” shows similar data for the second exposure condition, $n=4 \pm \text{Std. Dev.}$ * indicates that pTyr is significantly different from control (t-test, $p<0.05$).

Figure 3.

ScanSite motif analysis of Ser/Thr phosphorylation sites. Identified pSer and pThr phosphorylation sites were searched against the ScanSite database. The pSer/pThr phosphorylation sites that match each motif were summed and the averages are shown above. Black bars indicate untreated, grey bars indicate 250 μM Hg²⁺ and white bars indicate okadaic acid treated cells for panel A, n=3. Black bars indicate untreated, grey bars indicate 100 μM Hg²⁺ and white bars indicate pervanadate treated cells for panel B, n=4. * indicates that Hg²⁺ treatment was different from control and # indicates that okadaic acid treatment or pervanadate treatments were different from control using ANOVA followed by Tukey's test, p<0.05.

Figure 4.

Identified Try phosphorylation sites were searched against the ScanSite database. Matching pTyr sites for each motif were summed and the averages are shown. Black bars indicate untreated, grey bars indicate 250 μM Hg^{2+} and white bars indicate okadaic acid treated cells for panel A, n=3. Black bars indicate untreated, grey bars indicate 100 μM Hg^{2+} and white bars indicate pervanadate treated cells for panel B, n=4. * indicates that Hg^{2+} treatment was different from control and # indicates that pervanadate treatment was different from control using the Kruskal-Wallis test ($p < 0.05$).

Figure 5.

Adaptation of the KEGG B-cell signaling pathway including protection spectral counts. Boxes indicate pervanadate, 250 μM Hg^{2+} and 100 μM Hg^{2+} with the color intensity showing the average total spectral counts for that protection. Control samples had 2 or fewer spectral counts for all proteins shown. Proteins are labeled by their Uniprot entry name with the suffix “_MOUSE” removed. +p and -p indicate phosphorylation and dephosphorylation.

Figure 6.

Pervanadate and Hg treatment differentially increase pTyr. The number of Ascore filtered spectral counts for tyrosine phosphopeptides are shown, error bars indicate standard deviation. Control samples had no more than one spectral count for any of the sites shown. Black bars indicate pervanadate treated cells (n=4), grey bars 250 μM Hg²⁺ treated cells (n=3) and white bars represent cells treated with 100 μM Hg²⁺ (n=4). SH2 domains identified by Scansite are underlined.

Table 1a

Summary of phosphoproteins identified in and their changes in abundance in response to treatment

		Changed Compared to Control			
	Proteins	Proteins unique to Treatment	ANOVA (p<0.05)	>2 fold spectral count increase	>2 fold spectral count decrease
Control	420	19			
250 μ M Hg ²⁺	586	182	180	48	75
Okadaic acid	476	34	37	9	16
Control	418	29			
100 μ M Hg ²⁺	410	50	53	13	12
Pervanadate	469	125	160	42	49

Table 1b

Summary of phosphopeptides^a identified and their changes in abundance in response to treatment

		Changed Compared to Control				
	Phospho-Peptides	Peptides unique to treatment	ANOVA (P<0.05)	>2 fold spectral count increase	>2 fold spectral count decrease	Phospho Peptides %
Control	648	42				90.8
250 μ M Hg ²⁺	969	374	337	43	70	92.5
Okadaic acid	724	67	71	7	9	93.2
Control	600	85				97.6
100 μ M Hg ²⁺	596	70	65	7	7	98.0
pervanadate	691	211	212	45	31	98.0

^a All phosphopeptides were assigned to a protein in table 1a

Table 2

Ingenuity pathway analysis of proteins with altered abundance in response to treatment

Pathway Name	Proteins Identified	<u>100 μM Hg²⁺</u>		<u>250 μM Hg²⁺</u>		<u>Okadaic acid</u>		<u>Pervanadate</u>	
		Score ^a	#	Score	#	Score	#	Score	#
eIF2 Signaling	EIF3G, RPS3A, EIF3B, MAPK1, GRB2, MAPK3, EIF5, RPL22L1, RPLP2, RPLP0, RPLP1, EIF3D	2.21	3	3.95	8	1.78	2	6.88	11
B Cell Receptor Signaling	BLNK, BTK, PTPN6, CD79B, MAPK1, GRB2, MAPK3, PLCG2, SYK, CD22, GSK3A, CD79A	6.14	6	3.57	7	-	-	8.64	12
RAN Signaling	KPNA3, RANGAP1, RANBP2	-	-	3.51	3	-	-	-	-
Systemic Lupus Erythematosus Signaling	PTPN6, CD79B, MAPK1, GRB2, MAPK3, PLCG2, CD72, CD22, HLA-B, HNRNPC, CD79A	5.27	6	2.05	6	-	-	6.05	11
Phospholipase C Signaling	BLNK, BTK, CD79B, MAPK1, ARHGEF2, FNBP1, CD79A, PRKCB, SYK, HDAC1, MARCKS, MAPK3	5.18	6	3.28	8	-	-	5.90	11

^a Ingenuity reports a score that is the negative log (p-value)

Table 3

Ingenuity pathway analysis of pTyr containing proteins

Pathway Name	Proteins Identified	<u>100 μM Hg²⁺</u>		<u>250 μM Hg²⁺</u>		<u>Pervanadate</u>	
		Score ^a	#	Score	#	Score	#
B Cell Receptor Signaling	BLNK, BTK, PTPN6, MAPK1, PLCG2, SYK, CD79A, VAV1	6.1	3	10.3	6	7.87	7
14-3-3-mediated Signaling	TUBA1A, MAPK1, PRKCD, PLCG2, TUBA1C, TUBA1B	-	-	-	-	7.2	6
Remodeling of Epithelial Adherens Junctions	TUBA1A, MAPRE1, TUBA1C, TUBA1B	-	-	-	-	5.2	4
Axonal Guidance Signaling	TUBA1A, MAPK1, CFL1, PRKCD, PLCG2, TUBA1C, TUBA1B	-	-	-	-	5.0	7
Ephrin A Signaling	CFL1, VAV1	-	-	3.5	2	-	-

^aIngenuity reports a score that is the negative log (p-value)