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Low level Hg^{2+} exposure modulates the B-cell cytoskeletal phosphoproteome

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ABSTRACT

Exposure of Wehi-231 B-cells to Hg^{2+} for 5 min resulted in concentration dependent changes in protein phosphorylations. Phosphorylation was quantified using mass spectrometry to analyze TiO_2 and anti-pTyr antibody selected phosphopeptides from Wehi-231 digests. The most frequent and largest amplitude responses to Hg^{2+} exposure were increased phosphorylation although a decrease was observed for 1% of phosphoproteins detected in the untreated cells. A subset of proteins responded with an increase in phosphorylation to Hg^{2+} exposure at low micromolar concentrations. The majority of proteins required Hg^{2+} over 20 μM in order to increase phosphorylation. Ser/Thr phosphorylations are prominent in the cytoskeletal organization and the GTPase signaling systems and these systems are notable as the primary ones responding to the lowest concentrations of Hg^{2+}. Systems that required higher concentrations of Hg^{2+} to increase phosphorylation included immune receptor signaling. The proteins for which an increase in phosphorylation occurred at Hg^{2+} above 20 μM have a higher proportion of pTyr sites. Anti Ig stimulation of Wehi-231 cells confirmed that cytoskeletal protein phosphorylation and GTPase signaling are modulated in physiologically relevant B-cell receptor activation. Candidate kinases that respond to Hg^{2+} exposure at the low μM concentrations include MAP Kinase 1, CaM Kinase II delta and PAK2.

Significance: Mercury (Hg) is a wide spread environmental toxicant. Epidemiological and laboratory studies suggest that exposure to environmental Hg at current levels, which have been perceived to be non-toxic, may contribute to immune system dysfunction and autoimmune disease in humans and animals respectively. While we have previously shown that exposure of B lymphocytes to low levels of mercury interferes with B-cell receptor signaling mediated by post transcriptional phosphorylation events, overall the mechanism that is responsible for increased autoimmunity in mercury exposed human or animal populations is not well understood. The current study evaluated the dose dependent actions of mercury to change phosphorylation in the Wehi-231 cell line, an immature B-cell model in which actions of mercury on development of cell function can be evaluated. The study identified the cytoskeletal proteins as the most sensitive to modulation by mercury with changes in Ser/Thr phosphorylation being observed at the lowest concentrations of mercury. These findings indicate that the actions of mercury on B-cell immune function and development are at least in part likely mediated through changes in cytoskeletal protein phosphorylation.

1. Introduction

Epidemiological studies indicate that occupational exposure to environmental mercury (Hg) contributes to immune system dysfunction and autoimmune disease [1–6]. Furthermore, for populations that are exposed to environmental mercury through diet or air in non-occupational settings, several studies have found correlations between mercury blood levels and increases in pro-inflammatory cytokine levels, alterations in Ig isotype profiles and anti-nuclear antibody titers, all of which are associated with autoimmune disease [7–10]. A strong association between mercury exposure and autoimmune disease (AD) is also observed in animal models. Studies using rats and mice show that low level Hg^{2+} exposures can trigger a systemic lupus erythematosus (SLE)-like disorder referred to as Hg induced autoimmune disease (HgIA),

Abbreviations: AD, autoimmune disease; SLE, systemic lupus erythematosus; HgIA, Hg induced autoimmune disease; BCR, B-cell receptor; MRM, multiple reaction monitoring; FDR, false discovery rate; GO, gene ontology; ANOVA, analysis of variance; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PIANO, Platform for Integrative Analysis of Omics data

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reviewed in [11–14]. However, moving beyond animal models and epidemiology to a mechanistic understanding of the immunotoxic potential of Hg^{2+} has been difficult. Progress has been hindered by our lack of understanding of basic mechanisms in both humans and animals.

Historically there have been two long standing difficulties in studying mercury toxicity. First is the issue of mercury bio-reactivity. Both the divalent inorganic cation and the organic cations of mercury react avidly and rapidly with free thiol groups which are ubiquitous in free glutathione and proteins. Secondly, the binding is dynamic so the mercury can dissociate and jump from one protein thiol group to another. Binding of Hg^{2+} to thiols compromises the function of virtually any thiol containing protein when sufficient amounts of mercury are present, and reversibility of the binding is problematic for detection [15].

In order to address the complicated issues of Hg^{2+} bio-reactivity, we have previously employed mass spectrometry, coupled with bioinformatics to investigate the effect of Hg^{2+} on the B-cell phosphoproteome [16,17]. We concentrated on B-cells because of the similarity of HgIA to SLE, and the essential role played by auto-reactive B-cells in the pathology of a variety of autoimmune diseases, [18] especially SLE [19]. Our focus on the phosphoproteome builds on previous work of ours and others that used western blots to show that exposure to Hg^{2+} dramatically increases tyrosine phosphorylation in B-cells [20,21]. Our approach was to first utilize a discovery mass spectrometry strategy to determine how a short term exposure to relatively high concentrations of Hg^{2+} alters the global phosphoproteomic profile of B-cells [16,17]. These observations were subsequently validated with respect to specific proteins and phosphorylation sites by targeted analysis of selected proteins with multiple reaction monitoring (MRM), after exposure to lower and environmentally relevant concentrations of Hg^{2+} [16].

Our initial studies utilizing the discovery strategy resulted in the identification of specific phosphoproteins and signaling pathways that are likely to be molecular targets of Hg^{2+}. In particular, we identified a set of 119 proteins with modulated phosphorylation (p-value ≤ 0.05) after acute exposure to Hg^{2+}. Both DAVID [22,23] and Enrichr [24] pathway analysis algorithms identified the KEGG [25] B-cell receptor (BCR) signaling pathway as the pathway most significantly affected by Hg^{2+} exposure. The ExPlain (ExPlain 3.0 (http://biobase-international.com/)) key node algorithm identified the Src family tyrosine kinase Lyn as the most significant phosphoprotein regulatory node affected in Hg^{2+} intoxicated B-cells. Cytoscape (Reactome Functional Interaction plugin (2012 FI Network) of Cytoscape (ver 2.8.3)) analysis of the data also identified Lyn as a key node affected by Hg^{2+}, but indicated that aside from being part of the BCR signaling pathway, in Hg^{2+} exposed B-cells phospho-Lyn was also connected to the actin-cytoskeleton pathway [16]. Follow-up with targeted MRM analysis identified several Lyn phospho-sites, including the well-known dominant negative C terminal regulatory site, which were affected by exposure to environmentally relevant levels of Hg^{2+} [16].

Some time ago we demonstrated that membrane immunoglobulin likely interacted with the cytoskeleton [26]. More recent reports now show that BCR functionality is influenced by changes in membrane protein mobility mediated by phosphorylation changes to the cytoskeleton [27–30]. This suggests that exposure to Hg^{2+} could affect BCR function and B-cell activation through its effect on cytoskeletal protein phosphorylation. In order to evaluate this question we have utilized highly sensitive Orbitrap mass spectrometers to extend our initial observations on the effect of mercury on the B-cell phosphoproteome. The increase in instrument sensitivity has allowed us to utilize much lower Hg^{2+} concentrations than used in our initial investigation, [17] and has allowed us to more efficiently concentrate on the effect of mercury on the actin-cytoskeletal pathway in B-cells.

2. Materials and methods

2.1. Cell treatment

All treatments in this study were replicated four times as biological replicates with one week between each replicate. Wehi-231 cells were exposed to 0, 2, 5, 10, 20, 50 or 100 μM Hg^{2+} for 5 min. In a separate experiment the cells were treated with polyclonal goat affinity-purified antibody to mouse immunoglobulins (MP-Biomedicals-Cappel, catalog number: 55,441, Solon, OH), to activate the BCR complex. The activating antibody was added at 0.1 mg/ml and the time course for cellular response was measured by harvesting cells 0, 1, 2, 3, 5 or 15 min after activation of the BCR. The effective concentration range for the anti-BCR antibody was determined by titrating the reagent as an agonist for B-cell activation as previously described [31]. Cellular responses to the exposures were stopped by dispersing the cell suspension in ice cold HANK's solution. Cells were pelleted then solubilized in 1% LiDS and heated to 95 °C for 5 min to inactivate phosphatases [32]. Following filtration through a course filter (product no 89868, Thermo Fisher Scientific, Waltham, MA), samples were diluted 10 fold and digested overnight with sequencing grade trypsin. Phosphopeptides were selected by TiO_2 affinity selection and, separately, using anti-phosphotyrosine antibodies pY20 (Abcam, Cambridge, MA) and 4 g10 (EMD, Billerica, MA). Phosphopeptide enrichment strategies all have biases and no single technique is sufficient to completely map the phosphoproteome. This study combined the results from three phosphopeptide enrichment techniques, TiO_2 and the anti-phosphotyrosine antibodies pY20 and 4 g10, making it state of the art for maximum coverage in phosphopeptide selection. Despite this, it cannot be ruled out that some classes of phosphopeptides or phosphoproteins will be underrepresented in this analysis. All affinity selected samples were analyzed without further processing. All analyses were made using an Acclaim PepMap RSLC, 75 μm × 15 cm column with LC-MS/MS (liquid chromatography-tandem mass spectrometry) performed on a QExactive Orbitrap except for one replicate of phosphotyrosine immunoprecipitation samples which were analyzed on a Fusion Orbitrap mass spectrometer.

2.2. Protein and phosphorylation site identification

Tandem mass spectra were extracted by Proteome Discoverer version 1.4. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4), Sequest (Thermo Fisher Scientific; version 1.4.1.14) and X! Tandem (The GPM, the gpm.org; version CYCLONE (2010.12.01.1)). The Uniprot mouse complete database (downloaded 2014.06.02, 16,666 entries) was searched assuming the digestion enzyme trypsin. To ensure that each run was searched with the optimum mass tolerances, mass error was assessed in a test analysis and then search tolerances were set accordingly so that the fragment ion mass tolerance was either 0.020 Da or 0.60 Da and the parent ion tolerance was either 10.0 ppm (ppm) or 20 ppm. Carbamidomethyl modification of cysteine was specified as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and phosphorylation of serine, threonine and tyrosine were specified as variable modifications. In addition Glu to pyro-Glu of the n-terminus, ammonia-loss of the n-terminus and gln to pyro-Glu of the n-terminus were specified in the X! Tandem analysis as variable modifications. Scaffold (version 4.4.0, 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 > 99.0% probability. Peptide probabilities from X! Tandem, Sequest and Mascot were assigned by the Scaffold local false discovery rate (FDR) algorithm