

Electronic supplementary information

Title: Characterization of mercury-binding proteins in rat blood plasma

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Supplemental methods

Materials

Ribonuclease A (RA), carbonic anhydrase (CA), bovine serum albumin (BSA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), ethylenediaminetetraacetic acid (EDTA), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, USA). Iodine, potassium iodide (KI), and ammonium nitrate (NH_4NO_3) were obtained from Beijing Chemical Regents Company (China). NH_4OH was bought from Thermo Scientific (Rockford, IL, USA). Ultra-pure water was provided by a Milli-Q Advanced A10 system. All other reagents were analytical grade or better.

Animal treatments and blood plasma collection

Male Wistar rats were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The rats were acclimatized to the laboratory conditions for three days prior to the experimental exposure. They were housed in plastic cages under standard conditions of temperature (25 °C), humidity (< 70 %) and a 12 h/12 h light/dark cycle. Water and food were provided ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of Peking University and conducted according to the guidelines of care and use of laboratory animals.

For *in vivo* experiments, Wistar rats weighting ~300 g were randomly divided into four groups (n=3 of each group): (a) control group (drinking water); (b) low-dose HgCl_2 group (0.4 mg Hg kg^{-1}); (c) medium-dose HgCl_2 group (4 mg Hg kg^{-1}); (d) high-dose HgCl_2 group (20 mg Hg kg^{-1}). The animals were orally gavaged a single

injection of distilled water or mercury compounds. Blood samples from all groups were collected from the orbital venous plexus after 2 h of HgCl₂ exposure, while those samples from control and medium-dose group were collected after 2 h, 24 h, and 14 d after HgCl₂ exposure with heparin lithium as the anticoagulant. The whole blood was centrifuged at 2500 rpm for 10 min to separate the plasma and the red blood cells. The rats showed no behavioral abnormalities during the experiment.

***In vitro* incubation of rat blood plasma with mercury**

For the *in vitro* experiment, the plasma was spiked with HgCl₂ to a final Hg²⁺ concentration of 10, 100, 1000, and 10000 ng mL⁻¹, respectively. The plasma-mercury mixtures were incubated at 37 °C for 4 h. The plasma was aliquoted and stored at –80 °C until further analysis. The experiment was performed twice. To reduce the influence of long-term storage on mercury-binding proteins, the plasma samples were analyzed within one week following collection.

Measurement of total mercury in blood plasma

The concentration of total mercury in plasma was quantified with an Agilent 7500ce ICP-MS (Agilent, Palo Alto, USA) according to a previously described method.¹ Briefly, blood plasma samples were diluted into a 15-mL polypropylene Falcon[®] tube (Blue Max[™] Jr., Becton-Dickinson) with a diluent solution (1% NH₄OH, 2% g/L 1-butanol, 0.05% EDTA, and 0.05% Trion X-100).

GE-ICP-MS detection and mass spectrometry identification of mercury-binding plasma proteins

A modified commercially available Mini Prep Cell device (Bio-rad, Hercules, CA,

USA) equipped with a consecutive electrical power (DYY-10C, Liuyi, Beijing, China) was used for plasma proteins separation. The running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) was used for electrophoresis, while 50 mM NH_4NO_3 solution was used as elution buffer for transport of the effluent to ICP-MS. The experimental process was performed at 4 °C to maintain the stability of mercury-binding proteins to the highest extent.² Experiments were conducted following a previously described procedure with minor modifications.³ A reverse multilayer native gel (10 % → 8 % with lengths of 3 and 3 cm) was used for plasma protein separation and a 4 % native gel (1 cm) was used as the stacking gel. A three step voltage program was used for plasma proteins separation: 100 V for 10 min, 200 V for 90 min, and 600 V to the end.

The following I-markers with molecular weights ranged from 13 kD to 66 kD were used for determination of mercury-binding proteins molecular weights: RA (12 kD), CA (29 kD), and BSA (66 kD). These I-markers were prepared by labelling iodine to proteins. Briefly, the commercial standard proteins (RA, CA, and BSA) were dissolved in Tris-HCl buffer (0.1 mol L⁻¹, pH 7.5) to a final concentration of 1.0 mg mL⁻¹. The reactive iodination reagent, triiodide, was prepared by saturating KI solution (50 mmol L⁻¹) with iodine in Tris-HCl buffer (0.1 mol L⁻¹, pH 7.5). The standard protein solutions were incubated with the above resulting solution (5 mmol L⁻¹) for 5 min at room temperature. The reaction was terminated by adding sodium dithionite with a final concentration larger than 10 mmol L⁻¹. Small molecule inorganic salts in the iodine labelled proteins were removed by ultrafiltration (3000-

Da molecular mass cut-off). One known copper-containing protein in blood plasma, ceruloplasmin (132 kD, contains ~90 % of total copper present in plasma), was used as another marker to calibrate the molecular weights of mercury-binding proteins.⁴

Plasma protein samples (120 µg) were mixed with sample buffer (0.06 M Tris-HCl, 25 % glycerol, 2 % SDS, 3×10^{-4} M TCEP) in 2:1 (v/v) ratios and loaded onto the stacking gel. A T-connection tube was used to the outlet of column gel electrophoresis to split the effluent into two parts. One portion was transported to an Agilent 7500ce ICP-MS for elemental observation (Cu, Se, and Hg) and meanwhile in the other part mercury-binding proteins were collected into microvials for further protein identification.

The collected mercury-binding protein fractions were concentrated by 3000-Da molecular mass cut-off centrifugal concentrators (Millipore, Billerica, MA, USA), followed by one dimensional polyacrylamide vertical slab gel electrophoresis to further concentrate the target mercury-binding proteins. The 15 % resolving gel and 4 % stacking gel were used for separation of proteins with molecular weight less than 20 kD. Proteins with molecular weight ranged from 20 kD to 50 kD and larger than 50 kD was separated using 10 % resolving gel (4 % stacking gel) and 8 % resolving gel (4 % stacking gel), respectively. Electrophoresis was performed using a Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA, USA). Proteins were stained with silver staining for visualization. The mercury-binding protein lanes were excised from the silver-stained gels. The protein lanes were de-stained, reduced, rehydrated and vacuum evaporated, followed by incubated with sequencing-grade modified trypsin at 37 °C

overnight. The trypsin digestion peptides were analyzed by Micro TOF-Q II (Bruker Daltonics, Billerica, USA).

The MS/MS scan range window was set as 50-2200 m/z . The raw MS/MS data was searched against the database through Mascot 2.3.01 server. Search criteria were set as follows: one max missed cleavage, monoisotopic masses, peptide and fragment mass tolerance of ± 0.1 Da, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modifications. Protein hits were evaluated using a threshold of $p < 0.05$. Proteins with a minimum of three positive peptides per identification were regarded as positive.

The fraction of mercury bound to each protein was calculated as the contribute of the corresponding peak area to the sum of integrated peak areas.

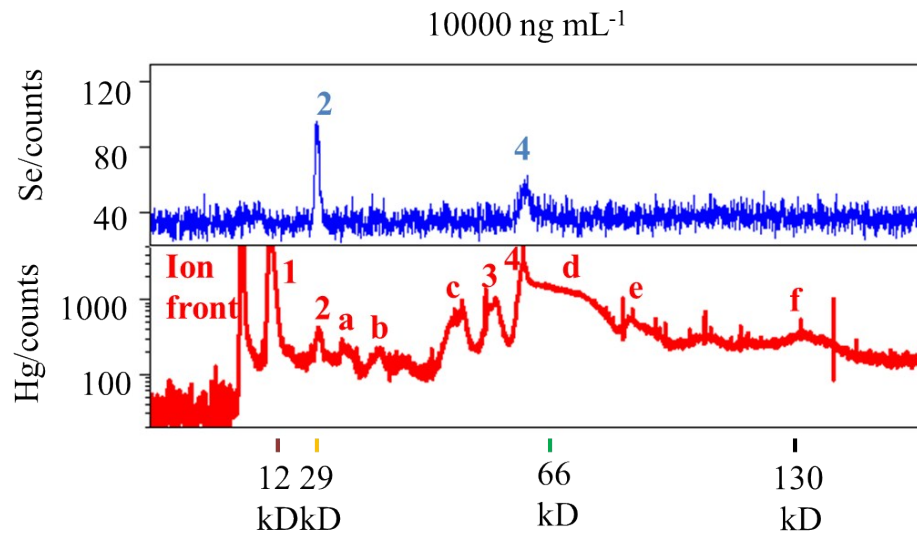


Fig. S1 The chromatogram of selenoprotein profile (blue line) and mercury-binding protein profile (red line) in rat blood plasma after 10 mg L⁻¹ of HgCl₂ incubation *in vitro*. Mercury-binding proteins: peak 1: hemoglobin; peak 2: Gpx3; peak 3: unidentified protein; peak 4: albumin/SelP; peak a: ApoA-I; peak b: ApoE; peak c: ApoA-IV; peak d: albumin; peak e: transferrin; peak f: unidentified protein.

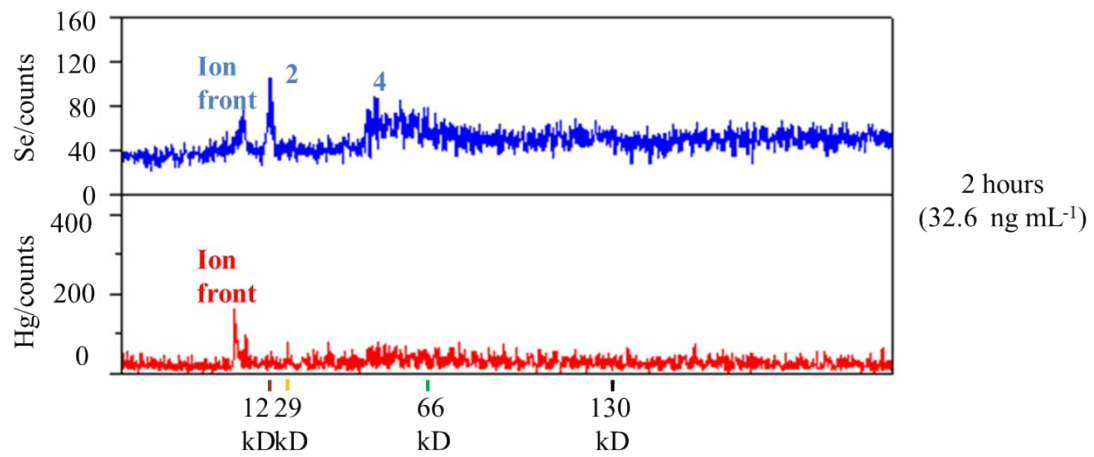


Fig. S2 The chromatogram of selenoprotein profile (blue line) and mercury-binding protein profile (red line) in rat blood plasma after 2 h of 0.4 mg Hg kg⁻¹ gavage.

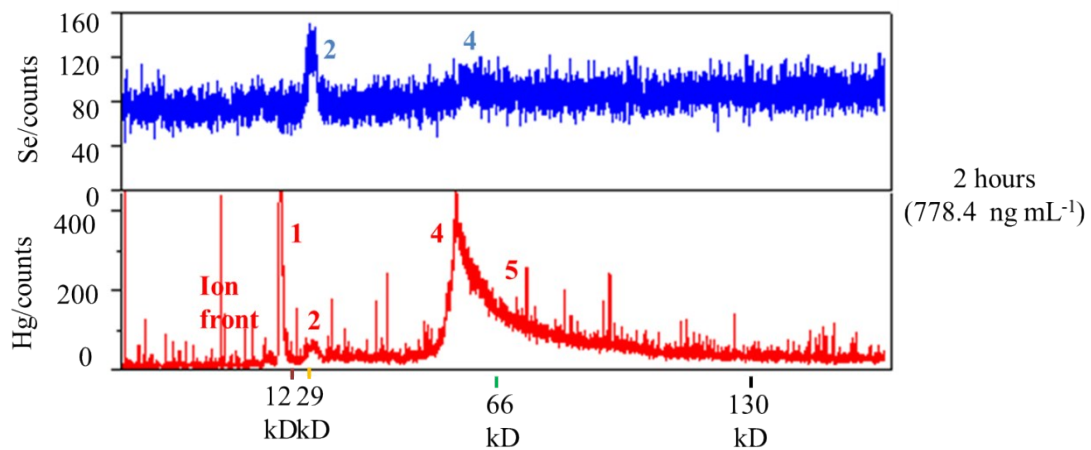


Fig. S3 The chromatogram of selenoprotein profile (blue line) and mercury-binding protein profile (red line) in rat blood plasma after 2 h of 20 mg Hg kg⁻¹ gavage. Mercury-binding proteins: peak 1: hemoglobin; peak 2: Gpx3; peak 4 and peak 5: albumin/SelP.

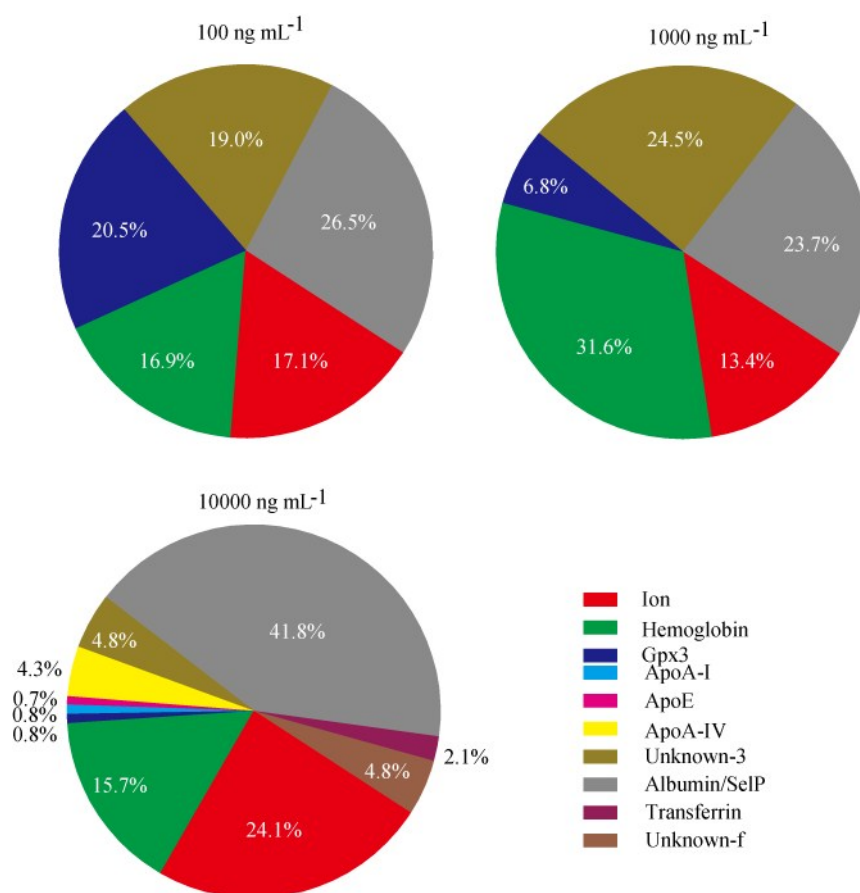


Fig. S4 The percentages of mercury present in different proteins in rat plasma after *in vitro* HgCl₂ treatment. The fraction of mercury bound to every protein was calculated as a percentage of the total sum of integrated peak area signals.

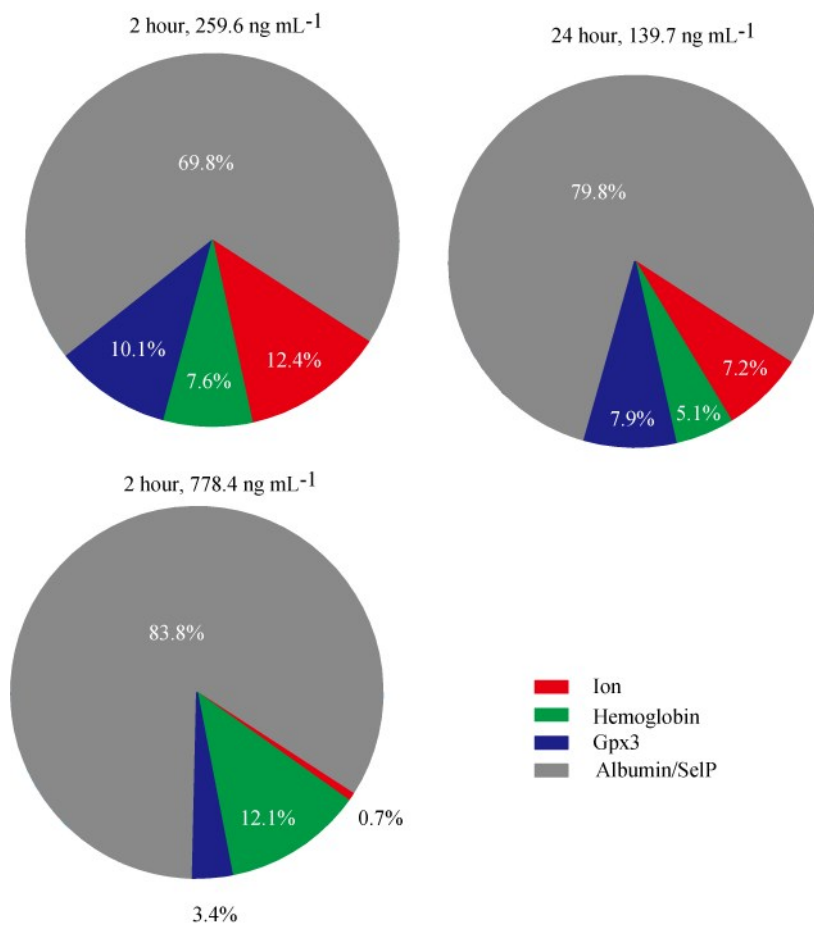


Fig. S5 The percentages of mercury present in different proteins in rat plasma after *in vivo* HgCl₂ treatment. The fraction of mercury bound to every protein was calculated as a percentage of the total sum of integrated peak area signals.

Table S1 Details of identified mercury-binding proteins in rat blood plasma.

Protein Name	UniProt AC	Molecular weight (kDa)/pI	Score	Protein Function
Hemoglobin	P02091	16.1/7.88	1343	Transfer/carrier protein
Glutathione peroxidase 3	P23764	25.6/8.26	82	Antioxidant enzyme
Apolipoprotein A-I	P04639	30.7/5.52	130	Circulating blood proteins
Apolipoprotein E	P02650	35.8/5.23	1185	Circulating blood proteins
Apolipoprotein A-IV	P02651	44.4/5.12	647	Circulating blood proteins
Selenoprotein P*	P25236	44.1/4.7	24	Peroxidase activity
Albumin	P02770	70.7/6.09	1451	Transfer/carrier protein
Transferrin	P12346	80/5.6	7831	Transfer/carrier protein

*MS/MS spectrum evidenced the successful identification of selenoprotein P with three peptides matched.

Reference

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