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Manganese and iron species in Sprague–Dawley rats exposed with $MnCl_2 \cdot 4H_2O$ (i.v.)

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ABSTRACT

Chronic manganese exposure leads to accumulation in brain mainly in the basal ganglia. Continuous incorporation finally causes neural damage (Manganism, Mn-PD) with disease pattern comparable to idiopathic Parkinson's disease (I-PD). Obviously, Mn is transported by an active mechanism across the blood–brain barrier (BBB) into the brain, but the exact transport mechanism is still not completely understood. The transferrin receptor shuttle is involved but it seems that this is not the only mechanism. Excretion of Mn follows slow simple diffusion. The exact transport mechanism is still unknown. Mn-metabolism involves a multiplicity of Mn species specifically affecting Mn-homeostasis. Low molecular mass (LMM) Mn species potentially facilitate Mn accumulation in brain.

Therefore, this paper investigated the formation and distribution of Mn- and Fe-Species *in vivo* (Sprague–Dawley rats) after a single intravenous (i.v.) injection of a non-toxic dose of $MnCl_2 \cdot 4H_2O$. Speciation analysis used a specifically developed sample preparation under cryogenic conditions and was performed with size exclusion chromatography online coupled to inductively coupled plasma-mass spectrometry (SEC-ICP-MS). The sample preparation method was previously shown to maintain species stability at enhanced extraction efficiency. One hour after i.v. injection overload of native Mn-carriers (Mn–transferrin) and formation of LMM Mn species, eluting at the retention time (RT) of Mn–citrate, were found in serum. After four days, serum and feces seemed to be Mn-cleared, but brain and kidney showed significantly elevated Mn levels. Even in lung and muscle tissue Mn was increased, but to a smaller extent. Accumulation of Mn especially in brain and kidney originated from increased concentrations of inorganic Mn and Mn bound to LMM Mn species. Iron status and iron species pattern appeared to be completely unaffected from i.v. injected Mn. The results underlined that specifically LMM-Mn-compounds permeated the BBB and accumulated in brain and kidney. It is discussed that kidney may play a regulatory role in the homeostasis of LMM Mn species.

1. Introduction

Manganese is an essential trace element [1,2] but also a potential neurotoxin emitted from occupational [3–5] and diffuse sources [6–12]. Chronic inhalation of Mn dust or aerosols leads to accumulation of Mn in specific target regions of the brain (Globus Pallidus, Caudate Putamen, White Matter, Cerebellum and Pituitary), but Mn accumulation was found also in kidney, lung, pancreas and bone [13]. Continuous Mn exposure finally causes neural damage in basal ganglia [14], called Mn-dependent Parkinsonism (Mn-PD) or manganism, having similar pathomechanisms to idiopathic Parkinson's disease (I-PD), because the same brain regions are affected [15].

At normal, physiological exposure Mn-homeostasis is well controlled by an efficient (99%) hepatobiliary excretion [16] causing a biological half-live of about 37.5 days [17] for healthy humans after oral administration or <24 h for rats [16] after i.v. injection. Human gastrointestinal absorption amounts to only 3% [18] of ingested Mn, but respiratory absorption of soluble Mn ranges from 60 to 85% [18,19]. In developed countries the average dietary intake of 1.4–9.0 mg Mn per day meets the recommended range of the "estimated safe and adequate daily dietary intake" (ESADDI) of 0.3 mg Mn for infants and up to 5 mg Mn for adults [20]. However, widespread human Fe deficiency is discussed as a promoter of Mn-absorption and thus supports increased Mn serum levels [21–24]. Accordingly, under Fe deficiency the dietary intake exceeds 20% of the total human Mn body burden of approximately 20 mg per day [2]. Together with inhalation of absorbable Mn this could increase Mn body burden considerably. Although Mn is known as an essential trace element, based on its neurotoxic properties and the acceptable daily intake (ADI) ranging only from 1.8 (women) to 2.3 (men) mg Mn per day [25], the Mn intake supposedly requires reduction.

Many organic Mn-compounds (Mn species) contribute to Mn-homeostasis *in vivo* [9–11,28,30]. High molecular mass (HMM) species, above all Mn^{3+}-transferrin, but also Mn^{2+}-albumin, Mn^{2+}-α_2-macro-

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and, to a lesser extent γ-globulin, represent the main Mn-carriers in serum [17,26]. Apo-transferrin specifically binds trivalent metal ions, therefore Fe^{3+} and Mn^{3+} could act as competitors at free metal-binding sites of Apo-transferrin [27]. Low dietary absorption, distribution (Mn^{3+}-transferrin), functionalizing (Mn-enzymes) and excretion of predominant HMM Mn species (above all Mn^{3+}-transferrin) reflects the "normal" first pathway of Mn-homeostasis in healthy subjects, which is strictly controlled due to efficient biliary excretion [16,17,26,28].

The second (different and) harmful pathway is described by high respiratory absorption of inorganic Mn that might overload the native HMM Mn-carriers and moreover can circumvent partly biliary excretion. Therefore, excess Mn in serum is still unbound or bound by low molecular mass (LMM) species which disturb Mn-homeostasis. It turned out that especially LMM Mn species can easily cross the blood–brain barrier (BBB) and facilitate Mn accumulation in brain [28]. Yokel and Crossgrove found a three-fold higher influx co-efficient from blood into brain for Mn–citrate compared to inorganic Mn or Mn–transferrin in Sprague–Dawley rats, using the *in situ* brain perfusion technique. However, Mn–citrate showed an up to 90% higher distribution in brain than Mn^{2+} or Mn–transferrin after direct injection into the parietal cortex, whereas brain–blood efflux of injected Mn showed even slower diffusion-driven ratios than the reference compounds sucrose and dextran [28], however, independent on the injected Mn species (Mn^{2+} or Mn–citrate). Brain Mn is influenced hence by facilitated uptake due to presumable carrier mediated transport of LMM Mn species across BBB and efflux of Mn species by slow simple diffusion. Increased biological half-lives of Mn in brain of rhesus monkeys (>45 days) [13] or rats (>58 days) [29] and accordingly humans might be explained by these processes.

Michalke et al. found Mn–citrate as the main Mn species in human cerebrospinal fluid (CSF) among other Mn species that could be related to the citric cycle [30,31]. Investigations of metal-species of paired human serum and CSF showed different species pattern and thus, suggests selective permeation of LMM Mn species across the blood–brain and blood–CSF barrier [32].

Metabolism of Mn is complex and involves a multiplicity of Mn–Species [31–33] with specific behavior *in vivo* [9,10,12]. Speciation analysis [34] of Mn several days after exposure of the experimental rats could improve understanding of both, the "native" and the "harmful" pathway of Mn within a living organism (rat) and especially the facilitated Mn-influx and accumulation in the brain [10,11].

With respect to our former findings gained from samples of non-exposed humans, the objective of this work was now to confirm (or withdraw) the roles of Mn species in passing neural barriers and accumulating in brain of still living organisms after low-dose exposure. For this purpose Mn-speciation and possibly interacting Fe-speciation were investigated. Mn–citrate was specifically of interest. Intravenous injection was chosen for Mn exposure, since the speciation and distribution in serum and various organs after low-dose exposure should be investigated, but not influences from different uptake pathways [35]. Mn- and Fe-speciation was performed in serum (1 h after i.v. exposure and after sacrifice (4 days)) and in various rat organs, providing improved insight into the roles of Mn species at neural barriers.

2. Materials and methods

2.1. Experimental design

Mn- and Fe-species are investigated in Sprague–Dawley rats after a single i.v. injection of Mn^{2+} ("+Mn") or isotonic saline ("−Mn", = controls). Rat serum was analyzed 1 h after i.v. injection of a non-toxic dose of Mn to get information about first formation of Mn species *in vivo* ("serum-[1 h]"). Brain, kidneys, liver, skeleton muscle tissue and serum ("serum-[4 d]") were analyzed after 4 days incubation. Thus, according to Takeda et al. [36], advanced whole-body distribution of Mn species and sufficient brain accumulation was supported. Total Mn- and Fe concentrations were determined with inductively coupled plasma-atomic emission spectrometry (ICP-AES) after pressure digestion of the biological samples. Characterizations of Mn- and Fe-species were performed with size exclusion chromatography online coupled to inductively coupled plasma-mass spectrometry equipped with a dynamic reaction cell (SEC-ICP-MS). Both, the specifically developed sample preparation under cryogenic conditions and the analytical methods ICP-AES/SEC-ICP-MS were previously evaluated and quality controlled with regard to extraction efficiency, species stability and long-term storage of labile native Mn species in rat sample matrices [37].

2.2. Chemicals

The chemical list consists of:

Tris (99.9%) from Roth, Karlsruhe, Germany. Bovine γ-globulin from Serva Electrophoresis, Heidelberg, Germany. Human serum albumin (HSA, 99%), bovine apo-transferrin (98%), manganese(II)chloride tetrahydrate (99.99%), sodium chloride (99.999%) metallothionein, citric acid, reduced and oxidized glutathione, histidine, and methionine, each from Sigma-Aldrich, Steinheim, Germany. NaCl (p.a.), NaOH (1 M), HCl (30%, suprapur), and HNO_3 (65%, subboiling distilled), each from Merck, Darmstadt, Germany. Iron(II)chloride tetrahydrate (98%), from Applichem, Darmstadt, Germany. Certified Reference Materials (CRM 184, 185, 186, and 191), each from the Institute for Reference Materials and Measurements, Geel, Belgium.

Certified custom assurance standard solutions XGLEN-1686/7, from Spex Certiprep, Metuchen, USA. Ar, Ar/H_2, and NH_3, each from Air Liquide, Gröbenzell, Germany. $N_{2\ liq}$ from Linde, Unterschleißheim, Germany.

2.3. Manganese-citrate/transferrin standards

Solutions of 1000 mg/L citrate or apo-transferrin and 100 μg/L Mn (0.36 mg/L $MnCl_2 \cdot 4H_2O$) in eluent A (see SEC, pH 7.4) were incubated for 24 h at 37 °C and stored at 8 °C. A sufficient formation of Mn–citrate and Mn–transferrin complexes were detected after one week of storage using online-coupling of SEC-ICP-MS.

2.4. Animals/sample collection

The animal experiments were approved by institutions IACUC and by Bavarian federal state government under the file number 55.2-1-54-2531-26-08. Three-month-old male Sprague–Dawley rats (450–550 g, Charles River WIGA GmbH, Sulzfeld, Germany) were housed pairwise with food and water ad libitum during 14 days of adaption time (22 ± 2 °C, relative humidity 50 ± 10%, 12/12 h light/dark cycle). A semi-purified diet Ssniff EF R/M AIN 93G (lot 8898321, Ssniff Spezialdiäten, Soest, Germany) with low levels of trace elements (48 mg Fe, 23 mg Mn, 40 mg Zn, 11 mg Cu each per kg dry weight) was fed in order to provide low baseline Mn body levels of rats before starting the experiments. Lowered levels of Mn and Fe promoted Mn resorption in previous studies [21,24].

After two weeks of adaption time the rodents were anaesthetized with isoflurane for a single intravenous (i.v.) injection into the tail vein of either 100 μL isotonic saline (0.9 g NaCl, ≥99.999%, in 100 g Milli-Q water, pH 6.5) for control rats or 100 μL of a solution, pH 6.5, at a dose of 1 mg Mn/kg body weight (BW), exactly prepared for each rat, individually related to its BW. Osmolarity of solutions was checked by an osmometer (Vogel OM801, Giessen, Deutschland).

During the entire experimental time the rats were housed in individually ventilated cages (IVCs) with cotton cloths and ammonia binding pads (Agrebe basic B5555,) Agrebe Pad Plus, DuoMedix OHG, Hamburg, Germany) as bedding. Thus, quantitative feces collection of each animal at 12 h periods was easily accomplished for 4 days experimental time.