The Uptake of Manganese in Brain Endothelial Cultures

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Abstract:
The present study focused on central nervous system (CNS) transport kinetics of manganese phosphate and manganese sulfate; these findings were correlated with the transport kinetics of manganese chloride ($MnCl_2$), a model Mn compound that has been previously studied. A series of studies was performed to address the transport of Mn salts in confluent cultured endothelial cells. The initial rate of uptake (5 min) of Mn salts (chloride, sulfate, and phosphate) in rat brain endothelial (RBE4) cell cultures is salt-dependent, with the highest rates of uptake for Mn chloride and Mn sulfate (as reflected by the greatest displacement of ^{54}Mn compared with control). Mn phosphate had a lower rate of uptake than the other two Mn salts. These data show that brain endothelial cells efficiently transport Mn sulfate.
Keywords: Central nervous system; Rat brain endothelial; Neurological disturbances

Article:
Introduction
Mn is an essential nutrient, which is especially critical during development (Hurley, 1981). Both the adult brain and the developing brain of the fetus normally contain a small amount of Mn (Cotzias et al., 1968). While Mn deficiency during development is associated with convulsive disorders (Papavasiliou, 1978; Papavasiliou et al., 1979; Tanaka, 1982), exposure to elevated levels of Mn in adulthood results in an irreversible brain disease characterized by prominent psychological and neurological disturbances (Cotzias et al., 1968; Barbeau et al., 1976).

Approximately 80% of Mn in plasma is bound to β_1-globulin and albumin (Foradori et al., 1967), and a smaller fraction of Mn is bound to transferrin (Tf). Mn binding to Tf is time-dependent (Aschner and Aschner, 1990; Aschner and Gannon, 1994; Aschner et al., 1999). When complexed with Tf, Mn is exclusively present in the trivalent oxidation-state (Aisen et al., 1969). At normal plasma Fe concentrations (0.9— 2.8 μg/ml), iron binding capacity (2.5-4 μg/ml), and Tf concentration (3 mg/ml, with two metal—ion-binding sites per molecule (M_r, 77,000), of which only 30% are occupied by Fe^{3+}) , Tf has 50 μmol/l of unoccupied Mn^{3+} binding sites. Tf receptors are present on the surface of the cerebral capillaries (Fishman et al., 1985; Jeffries et al., 1984; Partridge et al., 1987) where endocytosis of Tf is known to occur (Partridge et al., 1987). Support for receptor-mediated endocytosis of a Mn-Tf complex in cultured neuroblastoma cells (SHSY5Y) is provided by Suarez and Eriksson (1993).

Ligands for Mn^{2+} other than Tf must exist, however, because plasma that is saturated with Fe^{2+}, Zn^{2+}, and Cd^{2+} will bind Mn^{2+} (Scheuhammer and Cherian, 1985). A recent study (Dickinson et al., 1996) has addressed the tissue distribution of injected ^{54}Mn ($MnCl_2$) in hypotransferrinemic (Hp) mouse mutants. This mouse has a mutation in the Tf gene and produces <1% of normal (wild-type animals) Tf levels. ^{54}Mn accumulated at abnormally high levels in the Hp mouse liver, yet its distribution in the central nervous system (CNS) was not altered. These results reveal that Tf is probably required for proper targeting of Mn from the liver to other organs, but indicate that non-Tf transport mechanisms for Mn must also exist.

Unlike many other metals, Mn^{2+} does not possess high affinity for any particular endogenous ligand. There is almost no tendency for Mn^{2+} to complex to -SH groups or to amines. Not surprisingly, Mn^{2+} does not have

much variation in its stability constants for endogenous complexing ligands ($\log_{10}k$ = 3, 4, 3, and 3 for glycine, cysteine, riboflavin, and guanosine, respectively, where k is the affinity constant). It is likely that minute amounts of Mn^{2+} in plasma exist, according to the Mass Law Principal, as a chloride complex. While the amount of this complex in plasma at any one time must be minute, the Mass Law Principal states that a definite infinitesimal amount is always present. It also holds that if any Mn^{2+} should leave the plasma by dissolving in a lipid membrane, that the protein-Mn^{2+} complex should dissociate to maintain equilibrium.

An important process in the toxic outcome of metals is their transport from plasma into the brain across the capillary endothelial cells that comprise the blood-brain barrier (BBB). In order to cross this barrier, metal complexes must be either highly lipid soluble, or possess affinity for specific carrier-mediated transport systems within the endothelial cell plasma membrane. Little is known about manganese chloride transport, and virtually no experimental data exist regarding the transport mechanisms of manganese sulfate and phosphate across the BBB, a crucial step in Mn accumulation in the CNS.

Howand in what chemical form Mn is transported across the BBB remains controversial. It appears that facilitated diffusion (Rabin et al., 1993), active transport (Murphy et al., 1991; Aschner and Gannon, 1994; Rabin et al., 1993) as well Tf-dependent transport (Aschner and Gannon, 1994; Ueda et al., 1993) mechanisms are all involved in the transport of Mn across the BBB. Although, non-protein-bound Mn enters the brain more rapidly than Tf-bound Mn (Murphy et al., 1991; Rabin et al., 1993), the question remains as to which form represents the predominant mechanism of transport in vivo. At physiological Mn plasma concentrations, it is postulated that Mn transport across the BBB occurs both as a Tf-Mn conjugate (where Mn is in the 3+ oxidation -state) and as Mn^{2+}. Both are saturable transporters. A third transport system for Mn represents a leak pathway, presumably via circumventricular organs or choroid plexus. Since albumin or α_2-macroglobulin are excluded from transport across the BBB, Mn^{2+} bound to these plasma-binding proteins does not cross the BBB (unless the integrity of the BBB is compromised). As the plasma Mn concentration increases, Mn transport via the saturable transport systems and the leak pathway increases. However, since the transporters of Tf-Mn^{3+} conjugates and Mn^{2+} are saturable, their transport capacity is limited. Therefore, the leak-pathways become the predominant mode of Mn transport at high blood Mn concentrations, such as those described in bolus injection experiments.

Mn TRANSPORT IN CULTURED RAT BRAIN ENDOTHELIAL (RBE4) CELLS

The development of in vitro BBB models, consisting of cultured brain endothelial cells, has made possible the study of BBB transport phenomena at the cellular level. Basic characteristics of BBB transport of endogenous and exogenous solutes and their biochemical, pharmacological, ontogenic and pathological regulation mechanisms are, therefore, amenable to investigations.

Immunohistochemical and morphological assessments were carried out in RBE4 cells, kindly provided by Neurotech SA, Evry, France. The RBE4 cells were prepared from second-passage RBE4 cell primary cultures by transfection with the plasmid pE1A neo. This plasmid carries the entire E1A region of adeno-virus-2 conferring immortalization without oncogenic transformation, and the *neo* gene for resistance to the aminoglycoside G418 (geneticin; Durieu-Trautmann et al., 1993; Roux et al., 1993, 1994). These cells have been previously characterized for functional expression of the serotonin transporter (Brust et al., 2000), evoked acetylcholine release (Malo et al., 1999), Mrp1 multidrug resistance-associated protein and p-glyco-protein expression (Begley et al., 1996; Regina et al., 1998), γ-glutamyl transpeptidase and alkaline phosphatase activities (Roux et al., 1994), as well as production of vasoactive substances (Kis et al., 1999). These cells express cell markers specific to endothelium, including Factor VIII and angiotensin-converting enzyme activity, and low-density lipoprotein incorporation. Like endothelial cells in vivo, lateral membranes of adjacent cells overlap, and their plasma membranes form an extensive network of tight junctions, characteristic of the BBB. Notably, RBE4 cells, like cultured bovine brain endothelial cells, express transferrin receptors (TfR; Huwyler et al., 1999).

RBE4 cells were cultured and assessed for expression of Factor VIII. Briefly, the cells are seeded at a density of 10^3-10^4 cells/cm^2 on glass coverslips (for morphological characterizations) or six-well plates (for transport studies; see below) coated with rat-tail type-I collagen. The cells were grown in culture medium consisting of (α)-minimal essential medium/Ham's F10 (1:1 vol/vol; GIBCO), supplemented with 2 mmol/l glutamine, 10% heat-inactivated fetal calf serum, 1 ng/ml basic fibroblast growth factor, and 300 μg/ml geneticin (G418; Boehringer Mannheim) in humidified 5% CO_2/95% air at 37 °C. In the six-well cell culture plates the cells reach confluence after 2 days. The experiments were conducted at confluence with cell densities between 10^5 and 10^6 cells per well (~100-150 μg of protein). Immunohistochemical staining of the RBE4 cell monolayer was carried out with HRP-conjugated Factor VIII-related antigen (von Willebrand Factor, Dako Corp.), and attested to the endothelial nature and purity of the cultures.

A series of studies was performed to address the transport of Mn salts in confluent cultured endothelial cells (48 h post-plating). In control cultures, 0.3 μCi [^{54}Mn]-$MnCl_2$ was added to the medium and Mn uptake was determined at 5 min (DPM standardized for protein content, and expressed as 100%). In the other experimental conditions, Mn chloride, Mn sulfate, or Mn phosphate (each at 100 μM) were added to the brain endothelial monolayer simultaneously with 0.3 μCi [^{54}Mn]-$MnCl_2$, and $^{54}MnCl_2$ uptake was deter-mined (expressed as percent uptake versus control). The initial rate (5 min) of Mn uptake was salt-dependent, with the highest rates of uptake for Mn chloride and Mn sulfate (as reflected by the greatest displacement of ^{54}Mn compared with control); Mn phosphate exhibited a lower uptake rate. Similar studies were also conducted with 50 μM of the various Mn salts in the medium. Additional studies utilizing analogous techniques have further established that there are no qualitative differences in the absolute uptake and rank order of the various Mn salts between endothelial cells and astrocytes. Notably, both with 50 and 100 μM Mn salts, the rate (% Mn uptake versus control), and the rank order of transport of Mn salts were identical to those in astrocytes and bovine brain endothelial cells, with the highest transport for Mn chloride and Mn sulfate, and greater than Mn phosphate. Together, these data corroborate observations by Dorman (personal communication) that brain concentrations of Mn in rats repeatedly (14 days) exposed to Mn phosphate or sulfate (0-3 mg Mn/m^3) were significantly higher in the Mn sulfate-exposed group (no studies were conducted with inhaled Mn chloride).

Given the differential ability of the various Mn salts to displace $^{54}MnCl_2$ uptake in all the examined cell types, the data are inconsistent with mere diffusion of Mn, attesting to an active or carrier-mediated mechanism. We recognize that these results do not provide unequivocal proof for differential uptake of Mn in vivo; the only approach to prove or disprove the hypothesis of differential uptake of the various salts is to embark upon the in vivo studies. Though unlikely, it will also be important to establish in future studies whether the speciation of Mn in blood differs upon oral or inhalation exposure to various Mn salts.

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