Manganese Transport by Rat Brain Endothelial (RBE4) Cell-Based Transwell Model in the Presence of Astrocyte Conditioned Media

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Manganese (Mn), an essential nutrient, is neurotoxic at high levels and has been associated with the development of a parkinsonian syndrome termed manganism. Currently, the mechanisms responsible for transporting Mn across the blood–brain barrier (BBB) are unknown. By using rat brain endothelial 4 (RBE4) cell monolayers cultured in astrocyte-conditioned media (ACM), we examine the effects of temperature, energy, proton (pH), iron (Fe), and sodium (Na+) dependence on Mn transport. Our results suggest that Mn transport is temperature, energy, and pH dependent, but not Fe or Na+ dependent. These data suggest that Mn transport across the BBB is an active process, but they also demonstrate that the presence of ACM in endothelial cell cultures decreases the permeability of these cells to Mn, reinforcing the use of ACM or astrocyte cocultures in studies examining metal transport across the BBB. © 2005 Wiley-Liss, Inc.

Key words: energy dependence; proton dependence; iron dependence; sodium dependence; astrocyte-conditioned medium

Manganese (Mn) plays an important role in many biological processes. It is a component or cofactor of many enzymes involved in the metabolism of fats and proteins and is utilized by various antioxidant enzymes, such as superoxide dismutase (MnSOD) and glutamine synthetase (GS; Cotzias, 1958; Wedler and Denman, 1984; Lee, 2000; Takeda, 2003). Mn is involved in immune function, regulation of blood sugar, production of cellular energy, reproduction, digestion, bone growth, carbohydrate metabolism, and blood clotting (Aschner, 2000). Conversely, at high levels, Mn overexposure results in a clinical neuropsychological phenotype known as manganism, as well as motor symptoms often resembling Parkinson’s disease (Aschner and Aschner, 1991; Pal et al., 1999; Lee, 2000). Taken together, these data indicate that Mn readily crosses the blood–brain barrier (BBB), but the transporter(s) and/or protein(s) involved in this process is still a matter of debate.

The BBB, a semirestrictive barrier that provides the brain with an immunoprivileged status (Kniesel and Wolburg, 2000; Wolburg and Lippoldt, 2002; Engellhardt, 2003), is chiefly composed of endothelial cells in the vasculature (blood side), surrounded by astrocytic foot processes on the brain side of the barrier (Janzer and Raff, 1987; Isobe et al., 1996; Abbott, 2002). The permeability of the BBB is modulated by many factors, including the presence of the astrocytes. To date, however, the exact nature of the protein(s) or trophic factors regulating the intricate balance of permeability/impermeability of the BBB is not clearly understood. Nonetheless, in vitro studies suggest that the absence of astrocyte cocultures or astrocyte-conditioned medium (ACM) hinders the formation of tight junctions, which are observed in vivo between endothelial cells (Isobe et al., 1996; Kniesel et al., 1996; Kniesel and Wolburg, 2000; Wolburg and Lippoldt, 2002; Abbott, 2002; Engellhardt, 2003).

Further evidence supporting the role of astrocytes in formation of endothelial tight junctions has been reviewed (Aschner, 1998). For example, not only can type I astrocytes injected onto chick chorioallantoic membrane induce BBB properties in nonneural endothelial cells (Janzer, 1993) but expression of the barrier-specific marker GLUT-1 (glucose transporter-1) is down-regulated in brain capillary endothelial cells in the absence of astrocytes (Boado et al., 1994). Although

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other data demonstrate that the role of astrocytes is questionable (Rutten et al., 1987; Brightman, 1991; Holash et al., 1993; Holash and Stewart, 1993), it is still likely that astrocytes or ACM are necessary in vitro to produce cell culture models that closely mimic the properties of endothelial cells in vivo. Taken together, however, the data strongly suggest that it is necessary for astrocytes or ACM to be included in reliable endothelial culture models of the BBB.

For facilitating the transport of small molecules and lipophobic or charged particles across this semipermeable membrane, numerous transporters exist on the endothelial cells of the BBB. For example, the divalent metal transporter 1 (DMT-1; also known as DCT-1/NRAMP2) and the transferrin receptor (TfR) are known to be important in the transport of iron (Fe) into the brain (Rouault et al., 1985; Taylor et al., 1991; Malecki et al., 1999; Conrad and Umbreit, 2002; Garrick et al., 2003). Other data indicate that Mn$^{2+}$ is also transported by DMT-1 (Conrad and Umbreit, 2002; Yokel and Crossgrove, 2004) and that Mn$^{2+}$ forms a complex with transferrin in plasma that can be transported across the BBB in a manner similar to that of Fe (Aschner and Aschner, 1990; Malecki, 2001a; Malecki et al., 1999; Panic, 1967; Takeda et al., 2000). Mn is also found in plasma as Mn–citrate (Crossgrove et al., 2003; Yokel and Crossgrove, 2004). Although it is unknown how the Mn–citrate complex crosses the BBB, it is possible that the complex is a substrate for organic ion transporters (Friedrich et al., 2001; Friedrich et al., 2003) or the low-affinity choline transporter (Lockman et al., 2001). Because of the different oxidation states of Mn in vivo, and its ability to form multiple complexes, it is likely that no single transporter accounts for 100% of the Mn transported into the brain. Additionally, more information about the nature of Mn transport must be generated to identify further possible candidates for Mn transport.

To characterize Mn transport across the BBB better, we undertook a series of studies utilizing rat brain endothelial (RBE4) cells cultured in the presence of ACM. RBE4 cells originally derived from rat brain microvascular endothelium immortalized with a plasmid containing the E1A region of adenovirus 2 along with a neomycin-resistance gene (Durieu-Trautmann et al., 1993; Roux et al., 1994). These cells express an endothelial phenotype and, in the presence of astrocytes, exhibit differentiation characteristics of CNS endothelium (Roux et al., 1994; Begley et al., 1996; Rist et al., 1997). We and other groups have used RBE4 cells as a model of the BBB (Mroczkowska et al., 2000; Yang et al., 2001; Friedel et al., 2001; Calhau et al., 2002; Reichel et al., 2002; Yang and Aschner, 2003; Toimela et al., 2004), and here we examine the effect of changes in temperature, mitochondrial function, pH, and Fe or Na concentration on the permeability and flux of Mn in this in vitro model of the BBB to shed further light on the putative transporter(s) associated with Mn uptake into the brain.

## MATERIALS AND METHODS

### RBE4 Cells

Neurotech SA (Evry, France) graciously provided the RBE4 cells (Durieu-Trautmann et al., 1993; Roux et al., 1994). Cells were kept as monolayer cultures in collagen-coated T-75 flasks until confluent. Medium (see below) was changed after the initial 24-hr time period, and then every 2 days. Cell doubling time was about every 20 hr.

### Media Specifications

ACM, which is known to decrease transendothelial permeability (Yang et al., 2001; Yang and Aschner, 2003), was used for all the culturing and experimental procedures. ACM was made just prior to use. For preparation of ACM, type I astrocytes isolated from the cortex of newborn rats were grown to confluence. The medium from the astrocytes (minimal essential medium with Earl’s salts containing 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone; all from Gibco/Invitrogen, Carlsbad, CA) was collected and mixed with an equal volume of fresh RBE4 medium. The ACM was collected fresh from astrocyte cultures every 3 days, each time the medium for the RBE4 cells was changed. At the time of ACM collection, the astrocyte cell population was confluent. The ACM was added to RBE4 cell culture alpha-type medium, consisting of 50% (v/v) minimum essential medium (alpha medium), and 50% F-10 Nutrient Mixture (both from Gibco/Invitrogen). The alpha MEM/F10 medium was supplemented with 2 mM glutamine, 100 U/ml penicillin/100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum, and 300 μg/ml geneticin (G418; Boehringer Mannheim, Indianapolis, IN). On a per-use basis, 1 ng/ml basic fibroblast growth factor (bFGF; Invitrogen) was added to each new bottle of medium. The cultures were maintained in a humidified 37°C, 5% CO2/95% air incubator and fed 24 hr after subculturing, then fed as specified above (Regina et al., 1999; Yang and Aschner, 2003).

### Cell Culturing Technique

RBE4 cells were grown to confluent monolayers on porous, collagen I-coated, nitrocellulose filters. Cells were transferred to six-well plates, where they were eventually assayed. Each well had the porous filters attached to the base acrylic-walled inserts. The insert sat securely suspended in humidified 37°C, 5% CO2/95% air incubator and fed 24 hr after subculturing, then fed as specified above (Regina et al., 1999; Yang and Aschner, 2003).

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mine Mn concentration corrects for sequential removal of medium from the wells, new medium was not added to replace the aliquots.

Energy-Dependence Experiments

Energy dependence was determined by incubating confluent RBE4 monolayer for 2 hr in the absence or presence of three mitochondrial inhibitors (all from Sigma, St. Louis, MO), potassium cyanide (KCN, 4–5 mM), rotenone (10 μM), or oligomycin ABC (10 μM), at concentrations known to inhibit ATP production (Currie and Gregg, 1965; Slater, 1967; Riley and Winkler, 1990; Bizzozero et al., 1999). Polyethylene glycol (PEG) and dimethylsulfoxide (DMSO; 1:1 w/v) were used in stock preparations for rotenone in solution. PEG and DMSO did not disturb the monolayer integrity, corroborating earlier studies (Zalipsky et al., 1994; Blackstone, 2003; Gabizon et al., 2003).

Proton (pH)- and Sodium-Dependence Experiments

HEPES buffer was freshly made for each study at the specified pH levels. The Na\(^+\)-dependent studies utilized buffers with an isomolar substitution of choline chloride (Sigma) for NaCl and KOH for NaOH.

Iron (Fe)-Dependence Experiments

Prior to use, desferroxamine mesylate (DFO) or Fe-dextran (both from Sigma) were dissolved to 100 or 200 μM, respectively, in media or HEPES, pH 7.4, and neutralized with HCl or NaOH. Concentrations used here were consistent with previously published methodologies (Rouault et al., 1985; Breuer et al., 1995; Hasegawa et al., 1999) and within clinically relevant concentrations (Guo et al., 2002).

Analysis

The results are reported as mean ± SEM. All experiments were analyzed per well, with n ≥ 9. InStat (GraphPad Software, San Diego, CA) was used to analyze the data. When three or more conditions were compared, data were analyzed by one-way ANOVA, followed where appropriate, by post-test analysis. In instances in which only two treatments were compared, Student’s t-test was utilized. The type of analysis conducted is specified in the appropriate figure legends. Results were considered to be significantly different at P < 0.05.

RESULTS

Effect of Time and Temperature on Mn\(^{2+}\) Permeability and Flux

Initial experiments indicated that significantly less Mn (*P < 0.004) transversed RBE4 monolayers cultured in ACM compared with monolayers cultured in the absence of ACM at 120 min (Fig. 1). Although the time course for Mn accumulation in the bottom well was identical from 0 to 60 min, by 70 min a deviation between the two lines was observed. In light of these data, we wanted to characterize further the time- and temperature-dependence of Mn transport across RBE4 cells cultured in ACM. Figure 2 demonstrates that the cumulative mass of \(^{54}\text{Mn}^{2+}\) transversing RBE4 monolayers is a biphasic process under control (room temperature) conditions. Application of 0.6 nmol \(^{54}\text{Mn}^{2+}\) to RBE4 cells resulted in less than 15% (0.08 ± 0.003 nmol at 120 min) accumulation of the metal on the basolateral side of the control monolayers. In contrast, monolayers incubated at 4°C showed significantly less \(^{54}\text{Mn}^{2+}\) (0.0028 ± 0.0005 nmol at 120 min) accumulation on the basolateral side for all time points (n = 9, *P < 0.0001). This temperature-dependent decrease in basolateral Mn is consistent with an active transport process.

Effect of Mitochondrial Inhibition on Mn\(^{2+}\) Permeability and Flux

Mitochondrial inhibition was accomplished by treatment with KCN, oligomycin ABC, or rotenone. These compounds inhibit either complex I (KCN and rotenone) or complex V/F\(_{1}\)F\(_{0}\) ATPase (oligomycin ABC) of the electron transport chain. Figure 3 demonstrates cumulative transport of \(^{54}\text{Mn}^{2+}\) across RBE4 monolayers following preincubation with the respective inhibitory compounds. Compared with controls, treated monolayers showed significant reductions in \(^{54}\text{Mn}^{2+}\) permeability. Because rotenone is relatively insoluble under normal buffer conditions, resulting in a suspension, we also prepared a rotenone solution using PEG and DMSO to verify the ability of rotenone to inhibit \(^{54}\text{Mn}^{2+}\) permeability. The rotenone solution was unique in that maximal inhibition of Mn transport was observed by 5–10 min, whereas the
other compounds did not achieve maximal inhibition until approximately 20 min.

Effect of Proton (pH) Concentration on Mn²⁺ Permeability and Flux

To determine the effect of pH on Mn transport, monolayers were assayed in HEPES buffer at five pH levels: 7.0, 7.2, 7.4 (control), 7.6, and 7.8. As can be observed in Figure 4, control monolayers (pH 7.4) show ⁵⁴Mn²⁺ uptake to be a saturable process by 120 min, with a total of 0.25 ± 0.02 nmol ⁵⁴Mn²⁺ transported. At pH 7.0, ⁵⁴Mn²⁺ transport was significantly greater (n = 9, *P < 0.05) than control by 10 min, reaching 0.11 ± 0.01 (greater than 500% control). This trend continued throughout the remainder of the experiment (0.24 ± 0.01, 0.32 ± 0.01, and 0.40 ± 0.03 nmol at 30, 60, and 120 min, respectively) compared with controls values of 0.09 ± 0.007, 0.20 ± 0.01, and 0.25 ± 0.02 nmol, at the same time points (n = 9, *P < 0.05).

On the other hand, ⁵⁴Mn²⁺ transport was significantly lower at 30, 60, and 120 min for experiments conducted at pH 7.8 (n = 9, *P < 0.05). ⁵⁴Mn²⁺ transport at pH 7.2 or 7.6 was not statistically significantly different from control.

Effect of Fe Depletion or Supplementation on Mn²⁺ Permeability and Flux

Figure 5 shows the effect of Fe depletion or Fe supplementation on Mn transport. To deplete Fe levels in the culture media, monolayers were pretreated with 100 µM DFO in HEPES (pH 7.4). Fe chelation resulted in a significant increase (n = 9, *P < 0.05) in ⁵⁴Mn²⁺ transported across monolayers (0.24 ± 0.006 and 0.31 ± 0.008 nmol) at 60 and 120 min, respectively, representing increases of 27% and 22% over controls. Fe supplementation was achieved by incubating cell monolayers in 200 µM Fe-dextran in HEPES (pH 7.4). Increased Fe also resulted in a significant increase (n = 9, *P < 0.05) in ⁵⁴Mn²⁺ accumulation (0.22 ± 0.005 and 0.29 ± 0.004 nmol) at 60 and 120 min, respectively, representing increases of 16% and 15% over controls (0.093 ± 0.005 and 0.19 ± 0.008 nmol) at the same time points.

Fig. 2. Temperature dependence of Mn²⁺ carrier-mediated transport at the BBB. RBE4 monolayers were placed on ice, and transport and permeability were measured. There was a statistically significant decrease, as determined by Student’s t-test, in Mn transport in cells on ice compared with control cells at room temperature. Data are shown as average mass of Mn transversing the cell monolayers ± SEM (n = 9, *P < 0.0001).

Fig. 3. Energy dependence of Mn²⁺ carrier-mediated transport at the BBB. RBE4 monolayers were incubated for 1 hr with one of three mitochondrial inhibitors: KCN (4 mM), oligomycin ABC (10 µM), and rotenone in suspension (10 µM) or rotenone in a solution of PEG and DMSO (10 µM). All treatments resulted in statistically significant decreases in Mn transport (cumulative mol ± SEM) compared with controls by one-way ANOVA (n = 9, *P < 0.01 vs. control).

Fig. 4. Effect of pH on Mn²⁺ uptake. Monolayers were incubated in HEPES buffer at five pH levels: 7.0, 7.2, 7.4 (control), 7.6, and 7.8. There was a significant difference between the control and the other pH levels at 120 min (n = 9, *P < 0.05 vs. control).
Effect of Sodium (Na\(^{+}\)) on Mn\(^{2+}\) Permeability and Flux

To achieve a Na\(^{+}\)-free environment, RBE4 monolayers were allowed to equilibrate in Na\(^{+}\)-free buffer (as described in Materials and Methods) and immediately assayed for Mn permeability. Although the amount of \(54\text{Mn}^{2+}\) traversing the RBE4 monolayers in Na\(^{+}\)-free conditions at 10 min was statistically higher than control (0.054 ± 0.02 vs. control value 0.020 ± 0.001 nmol; n = 9, *\(P < 0.05\) vs. control). Relative to control (pH 7.4), Mn transport at pH 7.2 or 7.6 did not reach statistical significance.

**DISCUSSION**

Recent in vitro studies from our laboratory (Fitsanakis et al., 2005) concerning the transport of Mn across the BBB were conducted in the absence of ACM in the culture media. In order to mimic better the in vivo conditions, the major objective of this study was to characterize the nature of Mn transport across an in vitro model of the BBB providing optimal culture conditions for the RBE4 monolayer, more closely replicating the in vivo milieu. Initial results (Fig. 1) suggested that Mn permeability was greatly reduced when RBE4 cells were cultured in the presence of ACM. Thus, we wanted to characterize further the effect of temperature, mitochondrial inhibition, pH, and changes in Fe and Na concentrations on Mn permeability in cells cultured in the presence of ACM.

As can be observed in Figure 2, the effect of temperature is striking and immediate. These observations are consistent with carrier-mediated or channel-modulated transport and support the hypothesis that Mn influx across the BBB is not due to simple diffusion (Crossgrove et al., 2003). Further evidence supporting an active process is shown in Figure 3. The presence of mitochondrial inhibitors resulted in a dramatic decrease in Mn permeability. Although Mn itself may inhibit mitochondrial respiration (Gavin et al., 1992; Brouillet et al., 1993; Galvani et al., 1995; Zheng et al., 1998; Brown and Taylor, 1999; Malecki, 2001b), possibly contributing to the neurotoxic effects of the metal, Mn transport in the experiments reported here was attenuated by mitochondrial inhibition. Our data suggest that...
the initial [Mn] was insufficient to inhibit mitochondrial respiration completely and that the inhibitor concentrations were sufficient to reduce Mn transport. In that the brain [Mn] far exceeds that of the plasma (Keen and Zidenberg-Cherr, 1996; Keen et al., 2000), this lends credence to the hypothesis that Mn transport is an energy-dependent process, insofar as Mn must be transported against its concentration gradient. Our data support the premise that Mn transport is an energy-dependent and active process.

The data in Figure 4 indicate that Mn transport is pH dependent, suggesting several mechanisms for Mn transport. First, it is possible that Mn transport is facilitated by a H⁺-cotransporter functioning independently of ATP production or availability. This scenario is supported by the observation that decreases in pH lead to increases in Mn permeability. Second, perhaps ATP is necessary to facilitate the active transport of both H⁺ and Mn in an energy-dependent fashion, supported by data in Figure 3. Finally, Mn transport could be the result of two separate systems, one that is strictly H⁺ dependent and another that is strictly energy dependent. Currently, our data are insufficient to differentiate among the scenarios outlined above. The energy-dependent nature of Mn transport, taken together with the ability of Mn to modulate P2X receptors (North, 2002; Yamamoto et al., 2000), which pump ATP, mono- and divalent cations and larger moieties, suggests that Mn transport across the BBB may be more related to actual H⁺ currents and less related to simple proton stoichiometry.

We suspected, based on in vivo data from the Belgrade rat (Chua and Morgan, 1997; Burdo et al., 1999), microcytic mouse (Garrick et al., 2003; Lam-Yuk-Tseung et al., 2003), and hypotransferrinemic mouse (Dickinson et al., 1996; Takeda et al., 1998) models, all of which demonstrate a reduced capacity to transport both Fe and Mn because of either mutations in DMT1 or low levels of transferrin, respectively, that Mn transport would be Fe dependent. Additionally, we have shown that animals on an Fe-deficient diet achieved brain Mn levels similar to those of animals receiving a high-Mn diet with normal levels of Fe (Erikson et al., 2002). These in vivo data suggest high levels of Fe, as mimicked in vitro by Fe-dextrose, would lead to decreased Mn transport. Conversely, reduced Fe levels, achieved by treatment with DFO, would cause Mn transport to increase. This was not the case, as seen in Figure 5, but such results are not unprecedented in the literature for other in vitro systems (Crossgrove and Yokel, 2004; Yokel and Crossgrove, 2004).

It should be noted that dextran, like ascorbate and citrate, is a weak chelator of Fe and other metals, potentially allowing it to sequester and deliver metals to various transporters (May et al., 1999). Indeed, studies have shown that, among Mn²⁺, Mn-citrate, and Mn-transferrin, uptake was greatest for Mn-citrate (Crossgrove and Yokel, 2004), which would be most similar to an Mn-dextran complex. This may explain why we observed increased Mn transport in the presence of increased Fe.

The choline experiments (Fig. 6) clearly demonstrate that, in this model system, Mn transport is not Na⁺ dependent. Other work, however, suggests that Mn itself may be transported by the choline transporter (Lockman et al., 2001; Lockman and Allen, 2002; Allen and Lockman, 2003). Further studies examining the potential competition between Mn and choline would be beneficial. For example, studies utilizing bovine brain microvessel endothelial cells (bBMECs) indicated that doubling of [choline] results in a 50% increase in Mn²⁺ uptake (Crossgrove and Yokel, 2004). These data strongly suggest that, although Mn²⁺ transport may be temperature, energy, and pH dependent, it is not Na⁺ dependent. Future ion substitution experiments should target ions other than choline in order to prevent possible direct competition at choline transporters.

Finally, Table I compares data, presented as total Mn (nmol) transversing cell monolayers after 120 min, from RBE4 monolayers cultured in the absence or presence of ACM. In general, cultures with ACM demonstrate a greater impermeability to Mn compared with monolayers cultured in the absence of ACM. Indeed, the differences in permeability between the two groups
range from 3- to 100-fold. The one exception occurs in experiments in which pH is modulated; although pH does not statistically affect Mn permeability between groups (±ACM), it does modulate Mn permeability within the groups (see, e.g., Fig. 4). These data clearly demonstrate the dramatic effect that ACM has on modulating the permeability of the RBE4 cells to Mn. The fact that the inclusion of ACM in the culture media has a greater effect on permeability than any other condition tested cannot be overlooked.

In conclusion, the data indicate that Mn transport across RBE4 cell monolayers is an active process that is dependent on time, temperature, and pH. Although it is possible that the addition of ACM to RBE4 cell cultures may or may not reflect the in situ nature of Mn transport in the BBB, we propose that it is important to include either astrocyte cocultures or ACM in in vitro models of the BBB to mimic the in vivo environment of endothelial cells lining the BBB more closely. Such considerations should facilitate the generation of much needed information about the nature of heavy metal transport, particularly Mn transport, across the BBB.

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