Neurochemistry International 56 (2010) 843–849

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Effects of carboxylic acids on the uptake of non-transferrin-bound iron by astrocytes

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ARTICLE INFO

Article history:
Received 24 December 2009
Received in revised form 1 March 2010
Accepted 15 March 2010
Available online 23 March 2010

Keywords:
Citric acid
Malic acid
NTBI
Neurodegeneration
Tartaric acid

ABSTRACT

The concentrations of non-transferrin-bound iron are elevated in the brain during pathological conditions such as stroke and Alzheimer's disease. Astrocytes are specialised for sequestering this iron, however little is known about the mechanisms involved. Carboxylates, such as citrate, have been reported to facilitate iron uptake by intestinal cells. Citrate binds iron and limits its redox activity. The presence of high citrate concentrations in the interstitial fluid of the brain suggests that citrate may be an important ligand for iron transport by astrocytes. This study investigates whether iron accumulation by cultured rat astrocytes is facilitated by citrate or other carboxylates. Contrary to expectations, citrate, tartrate and malate were found to block iron accumulation in a concentration-dependent manner; α-ketoglutarate had limited effects, while fumarate, succinate and glutarate had no effect. This blockade was not due to an inhibition of ferric reductase activity. Instead, it appeared to be related to the capacity of these carboxylates to bind iron, since phosphate, which also binds iron, diminished the capacity of citrate, tartrate and malate to block the cellular accumulation of iron. These findings raise the possibility that citrate may have therapeutic potential in the management of neurodegenerative conditions that involve cellular iron overload.

1. Introduction

Iron is essential for normal cellular function and is required for mitochondrial energy metabolism, oxygen transport and DNA synthesis (Levenson and Tassabehji, 2004). However, excess unbound iron can promote Fenton chemistry and generate toxic free radicals that can damage cells (Halliwell and Gutteridge, 2007). The brain, with its high iron content and paucity of defence mechanisms, is particularly vulnerable to iron-mediated oxidative stress (Hallgren and Sourander, 1958; Heap et al., 1995). To minimise the potential for toxicity, iron is tightly bound to transferrin during transport and to ferritin during storage; both proteins bind iron in a redox-inactive form. In the brain however, the levels of transferrin in the interstitial fluid are relatively low (Bradbury, 1997; Ke and Qian, 2007; Moos and Morgan, 1998; Moos et al., 1999), and as a result, a substantial proportion of extracellular iron exists as non-transferrin-bound iron (NTBI). NTBI is generally present as Fe^{3+} and can form complexes with citrate, phosphate, ascorbate, albumin and ATP (Bradbury, 1997). The iron in these complexes is redox-active (Halliwell and Gutteridge, 2007), which is significant because NTBI levels in the brain increase in neurodegenerative disease (Connor et al., 1992; Dexter et al., 1991; Millerot-Serrurot et al., 2008).

Astrocytes are specialised for sequestering and detoxifying NTBI (Dringen et al., 2007; Hoepken et al., 2004), thus there is a need to understand the mechanisms they use to regulate NTBI levels in the brain. The divalent metal transporter 1 (DMT1) is used by intestinal epithelial cells for the absorption of Fe^{2+} from the diet (Mackenzie and Hediger, 2004; Tandy et al., 2000). While astrocytes express DMT1 (Jeong and David, 2003; Wang et al., 2001), DMT1-mediated iron transport is proton-dependent and requires an acidic pH (Garrick et al., 2006; Gunshin et al., 1997), so it is unlikely to be effective in the slightly alkaline pH of the interstitial fluid of the brain (Dringen et al., 2007; Kolokolova et al., 2008). Furthermore, DMT1 can only transport iron when it is in the Fe^{2+} form, so it must be coupled with a membrane-bound ferric reductase that can reduce Fe^{3+} to Fe^{2+}. However, the rate of membrane-bound ferric reductase activity displayed by cultured astrocytes is too low to account for their high rate of NTBI accumulation (Tulpule et al., 2010).

An alternate route for the uptake of NTBI is through the use of chaperone molecules, such as citrate, a tricarboxylic acid (tricarboxylate) that has a very strong binding affinity for Fe^{3+}

Abbreviations: CSF, cerebrospinal fluid; DMT1, divalent metal transporter 1; FIC, ferricyanide; FOC, ferrocyanide; IB, incubation buffer; LDH, lactate dehydrogenase; NTBI, non-transferrin-bound iron.

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doi:10.1016/j.neuint.2010.03.009

and a moderate affinity for Fe^{2+} (Martell and Smith, 1977). Citrate is used as a siderophore by bacteria, such as *Bradyrhizobium japonicium* and *Escherichia coli*, which take up iron as a ferric-citrate complex (Guerinot et al., 1990; Harle et al., 1995). In humans, citrate increases intestinal iron absorption from iron-supplemented drinks and meals (Ballot et al., 1987; Gillooly et al., 1983; Zhang et al., 2007). Cultures of the caco-2 human epithelial cell line have been used to show that citric acid (up to 200 μM) stimulates the cellular absorption of ferric iron (Salovaara et al., 2002). This stimulation is thought to occur because citric acid increases the solubility of Fe^{3+}, making it more readily available to proteins that transport Fe^{3+} (Salovaara et al., 2003). Interestingly, carboxylic acids including tartaric, malic, succinic and fumaric acid facilitate ferric iron uptake by caco-2 cells, similar to citric acid (Salovaara et al., 2002). Conversely, citric acid strongly inhibits the absorption of ferrous iron by caco-2 cells (Salovaara et al., 2002). This inhibition may occur because citric acid favours the oxidation of Fe^{2+} to Fe^{3+} (Bates et al., 1967; Konigsberger et al., 2000), thereby decreasing the pool of Fe^{2+} that is available for uptake via DMT1. Salovaara et al. (2003) suggest that the differential effects of citrate on Fe^{2+} and Fe^{3+} absorption indicate the existence of a separate transporter for Fe^{3+}.

Citrate is abundant in the brain where it is used for energy metabolism and lipid biosynthesis (Sonnewald et al., 2002), and it is present in the cerebrospinal fluid (CSF) at concentrations of 225–573 μM (Hoffmann et al., 1993; Michalke et al., 2007; Wishart et al., 2008). While it is not known whether citrate or other carboxylates can facilitate NTBI accumulation by astrocytes, this possibility is worthy of investigation given the evidence from non-neural cell types, and the presence of high concentrations of citrate in the CSF.

The present study has used primary cultures of rat astrocytes to investigate whether citrate or other carboxylates facilitate the accumulation of NTBI. The source of NTBI used in the present study is $FeCl_3$, a soluble form of Fe^{3+} that is readily taken up by astrocytes *in vitro* (Jeong and David, 2003; Takeda et al., 1998). Astrocytes were co-incubated with $FeCl_3$ plus a range of carboxylates, and their intracellular iron content measured. It was found that some carboxylates blocked the accumulation of iron. To determine whether this effect is due to an inhibition of membrane-bound ferric reductases, which might limit the amount of Fe^{2+} available for transport via DMT1, the cellular ferric reductase activity was measured. The results indicate that ferric reductase activity is not affected; instead, some carboxylates, such as citrate, appear to bind iron into complexes that cannot be taken up by astrocytes. These findings may have implications for neurodegenerative conditions such as stroke.

2. Materials and methods

2.1. Reagents

Dulbecco's-modified Eagle medium (DMEM), foetal calf serum (FCS) and penicillin/streptomycin was purchased from Gibco. $FeCl_3$ was purchased from Sigma, while potassium ferricyanide and potassium ferrocyanide were from ICN. The carboxylic acids (citric, tartaric, malic, succinic, fumaric, glutaric and α-ketoglutaric acid) were obtained from Sigma. When in solution at physiological pH, these compounds undergo deprotonation to become carboxylates (citrate, tartrate, malate, succinate, fumarate, glutarate and α-ketoglutarate) and will be referred to as such throughout this study. All other chemicals were obtained from Sigma or Merck.

2.2. Primary astrocyte cell cultures

Primary astrocyte cell cultures were derived from the brains of newborn Wistar rats (<24 h old), according to the procedure of Hamprecht and Loffler (1985). Viable cells were seeded in 24-well culture plates (300,000 viable cells/mL) and incubated in culture medium (90% DMEM, 10% FCS, 20 U/mL penicillin G, 20 μg/mL streptomycin). Cultures were maintained at 37 °C in 95% humidity and supplemented with 10% CO_2. The culture medium was replaced every 7 days and cultures were used for experiments after 14–17 days *in vitro*, with the culture medium being replaced at least 24 h prior to experimentation.

2.3. Incubation of cultures with $FeCl_3$

Cultures were washed twice with 1 mL of 37 °C incubation buffer (IB; 20 mM HEPES, 145 mM NaCl, 1.8 mM $CaCl_2$, 5.4 mM KCl, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 5 mM glucose, pH 7.4), and then incubated in 1 mL of incubation solution. The incubation solution was IB that contained 33 μM $FeCl_3$, and other reagents as detailed in the respective figure legends. Incubation of astrocytes with $FeCl_3$ produces accumulation in a linear manner for up to 6 h (data not shown). To control for the influence of the carboxylic acids, the pH of each incubation solution was adjusted to 7.4 following the addition of all reagents, and the pH was measured immediately prior to and after the incubation, to check for fluctuations in pH. Following the 3 h incubation, media samples were collected and stored at 4 °C, while cells were washed twice with 1 mL ice-cold phosphate-buffered saline (10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) and stored at −20 °C.

2.4. Cell viability

Cell viability was determined by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium following the incubation (Dringen et al., 1998). The protein content of untreated culture wells was determined using the Lowry protein assay (Lowry et al., 1951), and was used to standardise measurements of cellular iron content and ferric reductase activity between independent cell cultures.

2.5. Iron content

The iron content of cells and media was determined using a ferrozine-based colorimetric method described previously (Riemer et al., 2004). Cellular iron content for each culture well was standardised using the average protein content of untreated culture wells to give a measurement of nmol iron/mg protein. We have previously shown that washing the culture wells twice with PBS is sufficient to remove loosely-bound iron from the cell surface (Riemer et al., 2004), thus this procedure detects iron present within the cellular fraction. Media iron content was measured to ensure the accuracy of the iron concentration in the incubation media.

2.6. Ferric reductase activity

The ferric reductase activity of live astrocyte cultures was determined using an adaptation of the method of Lane and Lawen (2008). Cultured cells were incubated with 33 μM ferricyanide (FIC), a form of Fe^{3+} that cannot be taken up by cells, and the amount of ferrocyanide (FOC) that was produced as the cells reduced the FIC was measured. A 100 μL sample of media was taken immediately before and after the 3 h incubation, and transferred to a 96-well microtitre plate. 50 μL of 3.85% AcOH and 100 μL of developer solution were added to each well, and after 20 min the absorbance of the sample at 550 nm was measured. The developer solution contained four solutions, which were protected from light and stored at 4 °C for up to 3 weeks: (A) 3 M sodium acetate, adjusted to pH 6 using 10 M AcOH; (B) 0.2 M citric acid; (C) 3.3 mM $FeCl_3$ in 0.1 M AcOH; and (D) 20 mM ferrozine. The developer solution was prepared as required and contained solutions A, B, C and D in the ratio 2:2:2:1. The absorbance of samples was compared to the absorbance of FOC standards, to provide an indication of the concentration of Fe^{2+} in the samples. To calculate the amount of Fe^{2+} produced during the incubation, the $t = 0$ h media Fe^{2+} concentration was subtracted from the $t = 3$ h Fe^{2+} concentration for each culture well. The amount of Fe^{2+} produced in a cell-free control well was subtracted from each sample to control for any reduction of the FIC that occurred independently of cellular ferric reductase activity. The resulting values for each culture well were standardised to the average protein content of untreated culture wells, in order to quantify ferric reductase activity in each well (nmol iron/(h × mg protein)).

2.7. Statistical analysis

All experiments were performed in triplicate cell culture wells, on at least three independent cell cultures. The results shown are the mean ± SD of the independent cultures. The data were analysed using planned comparison independent samples *t*-tests to compare each experimental condition to the control condition within the same experimental replication. The significance was set at $\alpha = 0.05$. Where appropriate, *p*-values were adjusted using the false discovery rate correction (Benjamini and Hochberg, 1995) to control for the inflation of Type 1 error associated with multiple comparisons.

3. Results

To determine whether the presence of carboxylates affects the accumulation of NTBI, cultured astrocytes were incubated with 33 μM $FeCl_3$ plus concentrations of citrate, tartrate, malate, succinate, fumarate, glutarate or α-ketoglutarate that ranged from 1 μM to 1 mM. Following the 3 h incubation, cellular iron content was compared to that in control culture wells which had