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Iron uptake in quiescent and inflammation-activated astrocytes: A potentially neuroprotective control of iron burden

Ilaria Pelizzoni [a], Daniele Zacchetti [a], Alessandro Campanella [a], Fabio Grohovaz [a,b,*], Franca Codazzi [a,*]

[a] San Raffaele Scientific Institute, via Olgettina 60, 20132 Milano, Italy
[b] San Raffaele University, via Olgettina 58, 20132 Milano, Italy

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ABSTRACT

Astrocytes play a crucial role in proper iron handling within the central nervous system. This competence can be fundamental, particularly during neuroinflammation, and neurodegenerative processes, where an increase in iron content can favor oxidative stress, thereby worsening disease progression. Under these pathological conditions, astrocytes undergo a process of activation that confers them either a beneficial or a detrimental role on neuronal survival. Our work investigates the mechanisms of iron entry in cultures of quiescent and activated hippocampal astrocytes. Our data confirm that the main source of iron is the non-transferrin-bound iron (NTBI) and show the involvement of two different routes for its entry: the resident transient receptor potential (TRP) channels in quiescent astrocytes and the de novo expressed divalent metal transporter 1 (DMT1) in activated astrocytes, which accounts for a potentiation of iron entry. Overall, our data suggest that at rest, but even more after activation, astrocytes have the potential to buffer the excess of iron, thereby protecting neurons from iron overload. These findings further extend our understanding of the protective role of astrocytes under the conditions of iron-mediated oxidative stress observed in several neurodegenerative conditions.

1. Introduction

Astrocytes are versatile cells with a wide range of physiological functions. They have long been known to contribute not only to the formation of the Blood–brain Barrier (BBB) but also to provide trophic support to neurons and to regulate the synaptic microenvironment. More recently, they have been proposed to modulate neuronal activity and control neuroinflammation [1,2]. Within this complex framework, astrocytes participate to brain homeostasis of iron, an equally versatile element, which is essential not only for a wide variety of physiological functions but which is also able to induce oxidative damages when mishandled. Indeed, astrocytes control brain iron uptake through the BBB and are responsible for iron redistribution to neuronal cells. Several reports indicate that iron exceeds the binding capacity of Transferrin (Tf) in brain interstitial fluids, thus implying that a significant amount of iron circulates free or loosely bound to carrier molecules (e.g. ATP, ascorbate, citrate) released by the astrocytes [3,4]. This NTBI pool is considered to be the main source of iron for astrocytes in vivo, since the expression of Tf receptor 1 (TfR1) and the uptake of Tf-bound-iron (TBI) have been reported only in culture [5,6]. NTBI uptake was suggested to occur via the DMT1, the main transporter responsible for Fe^{2+} intake in mammals [7]; nonetheless, there is only limited evidence of its expression in vivo, at the level of the astrocytic perivascular endfeet [8,9], while its cellular distribution and specific plasma membrane localization in cultured astrocytes have yet to be clearly demonstrated [10,11]. Other mechanisms, such as the zinc transporter Zip14, have been proposed for NTBI uptake in astrocytes [7,12], but even in this case their physiological role is still to be established.

More recently, reports have shown that Fe^{2+} uptake can occur via calcium permeable channels in different cell types, a possibility that might have strong physiopathological implications not only for neurons but also for astrocytes [13–15]. The pathways responsible for NTBI uptake in astrocytes are still matter of debate: there is wide consensus that NTBI enters mainly as Fe^{2+}, while little evidence supports the existence of a Fe^{3+} import pathway in astrocytes in vitro [16,17].

Many studies indicate that the NTBI pool increases in pathological conditions: in acute brain injury, such as hemorrhagic stroke [18]; in several neurodegenerative disorders, causing the oxidative stress involved in disease progression [12,19]; in autoimmune diseases, such as multiple sclerosis [20]. All these pathological conditions are associated with neuroinflammation, a complex response to the cytokines and the pro-inflammatory molecules released by microglia. As a

Abbreviations: NTBI, non-transferrin-bound-iron; TRP, transient receptor potential; DMT1, divalent metal transporter 1; BBB, blood–brain barrier; Tf, transferrin; TfR1, transferrin receptor 1; TBI, Tf-bound iron; FAS, ferrous ammonium sulfate; FAC, ferric ammonium citrate; VOCCs, voltage-operated calcium channels; PLC, phospholipase C; DHPG, dihydroxyphenylglycine; IL, interleukin; TNF, tumor necrosis factor; LIP, labile iron pool; IFN, interferon; TIRF microscopy, total internal reflection fluorescence microscopy; EYFP, enhanced yellow fluorescent protein; NMDA, N-Methyl-D-aspartate; TRPV1, transient receptor potential vanilloid 1

* Corresponding authors at: San Raffaele Scientific Institute, Dibit, via Olgettina 58, 20132 Milano, Italy. Tel.: +39 02 26434811; fax: +39 02 26434813.
E-mail addresses: grohovaz.fabio@hsr.it (F. Grohovaz), codazzi.franca@hsr.it (F. Codazzi).

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consequence, also astrocytes undergo changes in their phenotype, in a process known as activation. Activated astrocytes surround brain lesions undergoing neurodegeneration and modulate the inflammatory response, with possible neuroprotective or detrimental effects on the neighboring neurons [21].

Although it is well established that inflammation influences systemic iron metabolism, little is known about the effects of neuroinflammation on brain iron homeostasis. In this study we characterize the mechanisms responsible for NTBI uptake in primary hippocampal astrocytes in resting conditions as well as upon inflammatory activation. Our final aim was to investigate whether the activation process could improve the capability of astrocytes to handle and buffer the NTBI pool, thereby protecting neurons by a potentially dangerous outcome [15].

2. Materials and methods

2.1. Cell cultures

Primary rat hippocampal astrocytes were prepared from 2 to 3 day-old Sprague–Dawley rats, according to [15]. The Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute approved the experimental procedures.

Pure astrocyte cultures were obtained by two steps of overnight shaking at 200 rpm; selective detachment of microglia was confirmed by the absence (<0.1%) of staining for IBA1, a specific microglia marker [22]. Confluent astrocytes were trypsinized and re-plated onto poly-lysine-coated coverslips or Petri dishes and experiments were performed within 3 days after re-plating. In order to obtain the activated phenotype, the astrocytes were treated with cytokines [23]. A mix of recombinant rat interleukin-1β (IL1β; 10 ng/ml) and tumor necrosis factor α (TNFα; 30 ng/ml) or interferon γ (INFγ; 20 ng/ml) was administered to astrocytes and incubated for 24 h at 37 °C. The cytokines were from R&D Systems (Minneapolis, MN, USA).

2.2. Videomicroscopy

The videomicroscopy setup is based on an Axioskope 2 microscope (Zeiss, GmbH, Martinsried, Germany) and a Polychrome IV (Till Photonics, GmbH, Martinsried, Germany) light source. The total internal reflection fluorescence (TIRF) microscopy setup was described in [24]. The ratio analysis was performed between the fluorescence signals (evaluated within the same region of interest) from TIRF and epifluorescence.

Fura-2 acetoxymethyl ester (Calbiochem, Merck KGaA, Darmstadt, Germany) and calcein acetoxymethyl ester (Molecular Probes, Life Technologies, Carlsbad, CA, USA) loadings were performed at 37 °C (4 μM 40 min and 0.25 μM 3 min, respectively) in Krebs Ringer Hepes buffer (KRH, containing 5 mM KCl, 125 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 6 mM glucose, 20 mM Hepes, pH 7.4). Single cell experiments were performed in KRH buffer at room temperature. To monitor Fe^{2+} variations, fura-2 was excited at 355 nm. This wavelength was adopted as isosbestic since it turned out to be Ca^{2+} insensitive in our optical configuration [15].

2.3. Pharmacological treatments

Fe^{2+} and Fe^{3+} water stock solutions were freshly prepared by dissolving ferrous ammonium sulfate and ferric ammonium citrate (Sigma-Aldrich, St. Louis, MO, USA), respectively. In some experiments cells were pre-treated for the specified times with pharmacological agents (Sigma-Aldrich) listed as follows: ebselen (50 μM, a DMT1 blocker), for 40 min; nimodipine (10 μM, a dihydropyridine L-type VOCC blocker), verapamil (100 μM, a phenylalkylamine L-type VOCC blocker), SC38249 (100 μM, a TRPC blocker) and LU52396 (10 μM, a TRPC blocker), for 15 min; oATP (100 μM, a blocker of P2X7 receptors) for 1 h.

2.4. Real time PCR analysis

RNA was extracted from cells with TRIzol (Invitrogen, Life Technologies) following manufacturer instruction. Single strand cDNA was obtained using Superscript III Retrotranscription Kit (Invitrogen) with random hexamers as primers. SYBR green-based reverse transcription quantitative PCR (RT-qPCR) was performed and analyzed on a LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Specific primers were: GTCCGATGGGGAAGAAGCA forward for DMT1-1A, CCT GGGATATGGGGTCGC forward for DMT1-1B, GTGAAGGGCTCCTCAGA ATC reverse for both DMT1 1A and B; GCCTGTCTGTCTGTCTTTGC and CCCAGTGTTTCCCAACTAACA for DMT1-IRE(+), TAGATGACCAACAG CCCAGA and CACAGCCGTTAGCTTTACCC for DMT1-IRE(−); TCACCA TTAAGCTGGGCG and TTCTTCCCGGTCCAGTCATA for frataxin (used for normalization).

2.5. LIP measurement

Cells were loaded with calcein and the fluorescence was measured before and after 15 min incubation with 100 μM salicylaldehyde isonicotinoyl hydrazone (SIH), a cell permeant iron chelator. The analysis was performed by using a High Throughput Microscopy (HTM) system, the IN Cell Analyzer 1000 [15] (GE Healthcare, Grandview Blvd, Waukesha, WI, USA).

2.6. ^{55}Fe uptake

To evaluate iron uptake, astrocytes (sampled in triplicate) were incubated 18 h with 2 μM ^{55}Fe–Ammonium Citrate (Perkin Elmer, Monza, Italy), corresponding to 2.5 μCi/ml, in the presence of 1 μM ascorbic acid (Sigma-Aldrich). For the higher Fe^{2+} concentration, 2 μM ^{55}Fe–Ammonium Citrate was mixed with 18 μM of non-radiolabelled Fe–Ammonium Citrate. Cells were then washed three times with phosphate-buffered saline and lysed with 20 mM Tris–HCl pH 7.4 with 0.5% Triton X-100. Cellular extracts were collected and centrifuged at 16,000 *g* for 10 min. Samples (10 μl) from the soluble fraction were mixed with 0.5 ml of Ultima Gold (Packard Instrument Co, Meriden, CT) and counted (3 min) in a scintillation counter (Packard Instrument Co). Finally, total protein content of soluble cellular extracts was used to normalize radioactive counts.

2.7. Expression vectors and cell transfection

The pEYFP-C1-DMT1-1A/IRE(+) and pEYFP-C1-DMT1-1B/IRE(+) vectors were generated as described in [25]. Primary hippocampal astrocytes were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were analyzed 24–48 h after transfection.

2.8. Western blotting

Cells were lysed by mechanical scraping in ice-cold PBS containing 0.1 mM EDTA, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate (SDS) and CLAP. Samples (20 μg of proteins per lane) in denaturating buffer (50 mM Tris/HCl, 2.5 mM EDTA/Na, 2% SDS, 5% glycerol, 20 mM DTT, 0.01% bromophenol blue) were incubated 10 min at 65 °C and proteins separated by standard SDS—polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto nitrocellulose membrane. Membranes were blocked with Tris–Buffered Saline (TBS) supplemented with 0.1% Tween-20 and 5% skimmed milk powder. Primary antibodies were diluted as follows: mouse anti-TfR1 antibody, 1:3000 and rabbit anti-actin 1:5000 (Invitrogen) in blocking solution; rabbit anti-DMT1 antibody 1:500 in TBS—0.1% Tween-20.