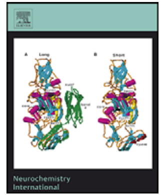
Neurochemistry International 55 (2009) 35–40

Contents lists available at ScienceDirect

Neurochemistry International

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



Selective up-regulation of GLT-1 in cultured astrocytes exposed to soluble mediators released by activated microglia

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A R T I C L E   I N F O

Article history:
Received 25 November 2008
Received in revised form 9 December 2008
Accepted 12 December 2008
Available online 7 February 2009

Keywords:
Microglia
Tumor necrosis factor
Glutamate transporter
Reactive astrocytes
dBcAMP
Neuroinflammation

A B S T R A C T

Impaired glial glutamate uptake is commonly involved in neuronal damages observed in acute and chronic nervous disorders. As nervous insults are frequently associated with local inflammation involving microglia, this study aims at exploring the link between activated microglia and altered glutamate uptake in astrocytes. The regulation of the expression and activity of type 1 glutamate transporter (GLT-1) was examined after exposing cultures of rat astrocytes to conditioned medium from lipopolysaccharide-activated microglia cultures. Significant increases in GLT-1 mRNA expression and dihydrokainate sensitive uptake of aspartate were observed after 72 h of treatment. These effects were reproduced by direct exposure of the astrocyte cultures to tumor necrosis factor alpha, a major cytokine released by activated microglia. The regulation of GLT-1 activity in response to inflammatory stimuli was also evidenced in cells exposed to dibutyryl cAMP, recognised as a model of reactive astrocytes in which the expression of this glutamate transporter is constitutively enhanced. Taken together, these results suggest that the GLT-1-dependent control of glutamate neurotransmission by either naive or chemically activated astrocytes is influenced by microglia-mediated inflammation.

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1. Introduction

During the last decades, glial cells have received considerable attention with respect to their putative role in the development and/or the progression of neurological disorders. Indeed, the innate immune protection of the CNS ensured by microglia might be compromised in pathological conditions, leading to a neurotoxic phenotype of activated cells with a robust inflammatory profile. Microglia activation is accompanied by the release of a variety of pro-inflammatory cytokines and free radicals which actively participate in the degenerative process (Kim and de Vellis, 2005; Block et al., 2007). Besides, excitotoxic neuronal damages frequently result from impaired handling of extracellular glutamate by astrocytes participating in gliosis. As microglia is recognised as a potent inducer of astrocyte activation, pro-inflammatory mediators are expected to modulate the glutamatergic transmission by regulating glutamate receptors and transporters (Tilleux and Hermans, 2008; Tilleux et al., 2007). Three major excitatory amino acid transporters (EAAT) subtypes termed GLAST (glutamate aspartate transporter), GLT-1 (glutamate transporter-1) and EAAC1 (excitatory amino acid carrier-1) have been cloned in the rat forebrain and are the rodent homologues of the human EAAT1-3, respectively (Danbolt, 2001). In astrocytes, GLAST is the predominant glutamate transporter during brain development, whereas GLT-1 becomes the most abundant subtype in mature organisms and is responsible for up to 90% of the total glutamate clearance in adult tissues (Tanaka et al., 1997). Regulation of GLT-1 has been extensively investigated, as it exhibits the highest affinity for the substrate and thereby efficiently contributes to protect neurons against excitotoxic insults. Hence, alterations in its expression or activity have been reported in several pathological conditions such as amyotrophic lateral sclerosis, multiple sclerosis or ischemia (Sheldon and Robinson, 2007). Consistently, several in vitro studies suggest that inflammatory mediators regulate glutamate transporters in cultured astrocytes (Tilleux and Hermans, 2007). Nevertheless, the interpretation of experimental data from functional studies is complicated by the co-expression of several transporter subtypes and differences in protocols for culture and maturation of astrocytes.

Culture of cortical astrocytes is a commonly used model for studying the regulation of glial glutamate transporters. These astrocytes are frequently grown in the presence of dibutyryl cyclic AMP (dBcAMP), in order to drive the cells into a differentiated phenotype (Schlag et al., 1998). These differentiated cultures are sometimes considered as activated astrocytes (Miller et al., 1994;

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doi:10.1016/j.neuint.2008.12.021

Tsugane et al., 2007) as they share several features with cells participating in reactive gliosis induced by lesions or inflammatory stimuli (Daginakatte et al., 2008). While astrocytes maintained under standard culture conditions (dBcAMP(−)) predominantly express GLAST, dBcAMP-exposed astrocytes (dBcAMP(+)) show enhanced expression of both GLAST and GLT-1, as commonly observed in several models of reactive astrocytes (Liang et al., 2008). With the aim to examine the influence of inflammation on the regulation of GLT-1, we herein studied the consequences of exposing cultured astrocytes to conditioned medium from lipopolysaccharide (LPS)-activated microglia or to the key inflammatory cytokine tumor necrosis factor-alpha (TNF-α). Both the expression of GLT-1 and the substrate uptake activity ensured by this transporter were studied in astrocytes grown in the absence or in the presence of dBcAMP.

2. Experimental procedures

2.1. Microglia-enriched and astrocytes-enriched cell cultures

Animal procedures were conducted in adherence to the European Community Council directives (86-609/EEC and 87-848/EEC). Mixed glial cultures were prepared from the cerebral cortices of 2-day-old Wistar rats, as described previously (Tilleux et al., 2007). After 10 days of proliferation (in Dulbecco's modified Eagle medium containing glutaMAX, supplemented with 10% foetal bovine serum, 50 mg/ml proline, 50 mg/ml penicillin-streptomycin and 2.5 mg/ml fungizone, all from Invitrogen, Merelbeke, Belgium), as cultures reached confluence, a microglia-enriched fraction was isolated by vigorous shaking (orbital shaker, 200 rpm). After 6 h shaking, detached microglial cells were collected by centrifugation at 1200 rpm during 5 min, and seeded in poly-L-lysine coated plates. As astrocytes contaminants which rapidly proliferate are frequently observed in these cultures, experiments (48 h medium conditioning) were initiated 24 h after plating. After 72 h, microglia represented >80% of the cells. Besides, cultures of astrocytes (>95% positive GFAP immunoreactive cells) were also obtained from the mixed glial cultures as the cells adhering to the flasks were submitted to another 12 h step of shaking to detach residual microglia and cell debris. Three days later, proliferating astrocytes were redistributed in poly-L-lysine coated multi-well culture plates and cell differentiation was initiated by decreasing the foetal bovine serum concentration to 3%. Where indicated, dBcAMP (150 μM) was added in order to trigger maturation/activation of the cells.

2.2. Microglial activation and astrocytes treatment

Twenty-four hours after plating, medium of microglia cultures was replaced by fresh medium containing 3% serum, as for mature astrocytes. LPS (1 μg/ml solubilised in culture medium) was added to half of the cultures. The cells were maintained under these conditions for 48 h in order to collect medium from activated or non-activated cells, respectively. After 48 h, the media were collected and transferred to the astrocyte cultures for 72 h. These conditioned media were previously shown to be enriched in pro-inflammatory cytokines and mediators (in particular, TNF-α, IL-1β, IL-6, PGE_2 and NO) (Tilleux et al., 2007). As control, some wells were incubated with culture medium that was kept in the incubator for identical periods of time but without microglial cells. Similarly, when testing the direct influence of LPS on astrocytes, fresh medium containing LPS was also incubated under identical conditions (without cells) before being transferred onto the astrocyte cultures. Where indicated, sister cultures were exposed to TNF-α (50 ng/ml, Sigma, Bornem, Belgium) or PGE_2 (1 μM, VWR international Europe, Leuven, Belgium).

2.3. RNA extraction, reverse transcription and real-time PCR

Total RNA was isolated using TriPure Isolation Reagent (Roche Diagnostic, Vilvoorde, Belgium) according to the manufacturer's protocol and reverse transcription was carried out with the iScript cDNA synthesis Kit (Bio-Rad, Nazareth, Belgium). Real-time PCRs were performed for the amplification of GLT-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) using the following primers: GAPDH forward 5′-GTCTCCTGTGACTTCAACAG-3′, reverse 5′-AGTTGTCATTGAGAGCAATGC-3; GLT-1 forward 5′-GCCAATACCAAGGCAGT-3′, reverse 5′-TTCATCCCGTCCTTGAACTC-3′. The real-time PCRs were carried out in a total volume of 25 μL containing 2 ng cDNA template, 0.3 μM of the appropriate primers and the IQ™ SYBR® Green Supermix 1x (Bio-Rad). The protocol implied 40 amplification cycles (15 s denaturation at 95 °C, 45 s annealing at 60 °C and 15 s elongation at 79 °C) and was performed using an iCycler IQ™ multicolor Real-Time PCR detection system (Bio-Rad). For quantification, relative standard curves were determined as previously described for other transcripts (Tilleux et al., 2007).

2.4. Immunoblotting

Astrocytes grown in 6-well plates were rinsed and scraped in phosphate buffer saline (PBS, 137 mM NaCl, 21 mM $NaHPO_4$, 29 mM KH_2PO_4, 1.2 mM KCl, pH 7.4). After centrifugation at 14,000 rpm for 5 min, proteins were solubilised in the solubilisation buffer (10 mM Tris–HCl pH 7.4, 20 mM CHAPS, 0.5 mM EDTA, 30 mM DTT, 0.5 mM PMSF, 1 μM protease inhibitor cocktail). After measuring the protein concentration, each sample was diluted in the loading buffer (125 mM Tris–HCl pH 7.4, 50 mM dithiothreitol, 4% sodium dodecylsulphate, 20% glycerol, 0.01% bromophenol blue, pH 6.8) and stored at −20 °C. For immunoblotting analysis, total protein extracts were boiled for 5 min, electrophoresed through a 7.5% sodium dodecylsulphate-polyacrylamide gel and transferred to nitrocellulose membranes by electroblotting. To avoid non-specific immunodetection, membranes were incubated for 1 h in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 5% non-fat milk. Immunoprobing was performed using affinity-purified antibodies recognising GLT-1 (Chemicon international, Hampshire, United Kingdom, 1/2500) and actin (Sigma, 1/750). Antigen-antibody complexes were detected with a horseradish peroxydase-conjugated goat anti-guinea pig IgG (for GLT-1, 1/5000, Jackson Immunoresearch Laboratory, DePinte, Belgium) or goat anti-rabbit IgG secondary antibody (for actin, 1/3000, Sigma). The immunoreactive proteins were detected with enhanced chemiluminescence reagents followed by autoradiography. For quantification, autoradiograms were analysed by densitometry using an MCID-M4 imaging system (Imaging Research, Ontario, Canada).

2.5. Aspartate uptake assay

The activity of the glutamate transporter was measured as previously described (de Hemptinne et al., 2004), using [^{3}H]-D-aspartate (50 nM, Perkin Elmer, USA) as substrate. D-aspartate is commonly used because it does not efficiently activate glutamate receptors and is a non-metabolizable analogue of glutamate. The specific uptake velocity (measured after 6 min) was estimated after subtracting the values obtained using Na^+-free Krebs buffer. Where indicated, 100 μM DHK (Ocean Product International, Canada), inhibiting GLT-1 activity, was added 6 min before the addition of the substrate.

2.6. Statistical analysis

Unless otherwise stated, statistical analyses were performed using one-way ANOVA, followed by Dunnett's post-test for comparison with the corresponding control and p values <0.05 were considered significant. When experiments were conducted with astrocytes grown in the presence or in the absence of dBcAMP, statistical analyses were performed separately.

3. Results

3.1. Modulation of dihydrokainate (DHK)-sensitive aspartate uptake in astrocytes exposed to conditioned medium from activated microglia

Focusing on the involvement of GLT-1, the influence of mediators released by activated microglia on the excitatory amino acid transport in astrocytes was investigated by measuring the Na^+-dependent uptake of D-[^{3}H]-aspartate, used at a tracing concentration of 50 nM. Indeed, compared to GLAST, GLT-1 is modestly expressed in cultured astrocytes, but thanks to its high affinity for glutamate and aspartate, its detection is reinforced at low substrate concentrations. Under these conditions, approximately 20% of the aspartate uptake (which reaches 7.5 pmol/mg prot/min) was inhibited when measured in the presence of the selective GLT-1 inhibitor DHK (100 μM). In order to specifically examine the regulation of GLT-1, our data were analysed by considering the DHK-sensitive fraction of aspartate uptake calculated by subtracting the values corresponding to the DHK-resistant fraction from the values of total aspartate uptake. In astrocytes grown in standard conditions, the medium from activated microglia significantly increased (by 43% after 72 h) the DHK-sensitive aspartate uptake (Fig. 1A).

As previously documented (Schlag et al., 1998), the total activity of glutamate uptake in these cells was enhanced in dBcAMP(+) astrocytes (reaching up to 14.8 pmol/mg prot/min when examined with 50 nM substrate), and the DHK-sensitive uptake accounted for approximately 1.9 pmol/mg prot/min,

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