The International Journal of Biochemistry & Cell Biology 41 (2009) 2599–2609

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Organic cation/carnitine transporter OCTN3 is present in astrocytes and is up-regulated by peroxisome proliferators-activator receptor agonist

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ARTICLE INFO

Article history:
Received 6 May 2009
Received in revised form 26 August 2009
Accepted 30 August 2009
Available online 6 September 2009

Keywords:
OCTN transporters
PPARα
Astrocytes
Carnitine
Peroxisomes

ABSTRACT

In the brain β-oxidation, which takes place in astrocytes, is not a major process of energy supply. Astrocytes synthesize important lipid metabolites, mainly due to the processes taking place in peroxisomes. One of the compounds necessary in the process of mitochondrial β-oxidation and export of acyl moieties from peroxisomes is L-carnitine. Two Na-dependent plasma membrane carnitine transporters were shown previously to be present in astrocytes: a low affinity amino acid transporter $B^{0,+}$ and a high affinity cation/carnitine transporter OCTN2. The expression of OCTN2 is known to increase in peripheral tissues upon the stimulation of peroxisome proliferators-activator receptor α (PPARα), a nuclear receptor known to up-regulate several enzymes involved in fatty acid metabolism. The present study was focused on another high affinity carnitine transporter—OCTN3, its presence, regulation and activity in astrocytes. Experiments using the techniques of real-time PCR, Western blot and immunocytochemistry analysis demonstrated the expression of *octn3* in rat astrocytes and, out of two rat sequences ascribed as similar to mouse OCTN3, XM_001073573 was found in these cells. PPARα activator–2-[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]acetic acid (WY-14,643) stimulated by 50% expression of *octn3*, while, on the contrary to peripheral tissues, it did not change the expression of *octn2*. This observation was correlated with an increased Na-independent activity of carnitine transport. Analysis by transmission electron microscopy showed an augmented intracellular localization of OCTN3 upon PPARα stimulation, mainly in peroxisomes, indicating a physiological role of OCTN3 as peroxisomal membrane transporter. These observations point to an important role of OCTN3 in peroxisomal fatty acid metabolism in astrocytes.

1. Introduction

There is a strong metabolic interaction in the brain between astroglial cells and neurons, resulting from the spatial distribution of enzymes and transporters, often expressed only in one type of these highly specialized cells. Astrocytes are not only responsible for the removal of neurotransmitters and delivery of their precursors, as in case of a so-called glutamate/glutamine cycle (Bak et al., 2006), but also for supplying neurons with important nutrients. Although glucose is acknowledged as the main energetic substrate of resting neurons, lactate released by astrocytes can be the other energy source upon neurons activation (Magistretti and Pellerin, 1999; Pellerin and Magistretti, 2004). On the contrary to peripheral tissues, fatty acid β-oxidation has not been considered an important process in the brain and, as shown by ^{13}C nuclear magnetic resonance spectroscopy, this process, contributing by 20% to brain energy production, takes place in astrocytes (Ebert et al., 2003). There are, however, other important steps in fatty acid metabolism which take place in astrocytes, just to mention cholesterol synthesis (Nieweg et al., 2009), as well as the elongation and desaturation of long-chain polyunsaturated fatty acids, delivering precursors for biologically active compounds, as prostaglandins, leukotriens, etc. The metabolic pathways of etherphospholipids biosynthesis, β-oxidation of very long-chain fatty acids and α-oxidation have been

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; OCTN, organic cation transporter novel family; PBS, phosphate buffered saline; PPAR, peroxisome proliferators-activator receptor; SDS, sodium dodecyl sulfate; TRITC, tetramethyl-rhodamine isothiocyanate; WY-14,643, 2-[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]acetic acid.

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doi:10.1016/j.biocel.2009.08.020

localized to peroxisomes (Wanders, 2004). These organelles are as well a site of one round of β-oxidation leading to the formation of a long-chain polyunsaturated fatty acid, docosahexaenoic acid (C22:6), a compound not only enriched in brain phospholipids but also known to be increased in ischemia (Farias et al., 2008) and after traumatic brain injury (Pilitsis et al., 2003), as well as to act as anti-inflammatory factor (Orr and Bazinet, 2008).

The lipid compounds metabolized in peroxisomes have to cross peroxisomal membrane, anyhow, the mechanism of such a transport remains obscure. One of the compounds necessary for the transfer of acyl moieties through the membranes is L-carnitine (4-trimethylammonio-3-hydroxybutyrate). Although carnitine concentration in the brain is lower than in peripheral tissues and plasma (Shinawi et al., 1998), it accumulates in the brain (Bresolin et al., 1982; Kido et al., 2001), due to the activities of carnitine transporters in the blood–brain barrier (Czeredys et al., 2008; Berezowski et al., 2004; Miecz et al., 2008). In mammals there are two families of transporting proteins involved in carnitine uptake: the first one, comprises the organic cation novel family (OCTN) transporters coded by solute carrier family 22 genes (*SLC22*), the second one is represented by an amino acid/carnitine transporter $B^{0,+}$ (belonging to transporters coded by *SLC6* gene family). The $B^{0,+}$ transporter catalyses uptake of neutral and positively charged amino acids in a Na^+ and Cl^- dependent way (Sloan and Mager, 1999) and it has also been shown to transport carnitine with a low affinity (Nakanishi et al., 2001). Hitherto, there are known three members of OCTN family. There are several controversies concerning OCTN1, as regards its ability to transport carnitine and it was shown recently to be ergothioneine transporter without carnitine transport activity (Gründemann et al., 2005). OCTN2 and OCTN3 are known as high affinity carnitine transporters (Schömig et al., 1998; Sekine et al., 1998; Tamai et al., 1998, 2000; Wu et al., 1998). Apart from their high sequence homology and predicted structural similarities, OCTN2 and OCTN3 differ in their functional characteristics. OCTN2 was shown to transport both, carnitine and organic cation—tetraethylammonium in a Na^+-dependent and Na^+-independent way, respectively (Tamai et al., 1998, 2000), while OCTN3 was demonstrated to transport carnitine independently of a [Na^+] gradient and was not capable of transporting tetraethylammonium (Tamai et al., 2000). In peripheral tissues OCTN3 has been shown to be localized in peroxisomes and was postulated to be a peroxisomal membrane transporter (Lamhonwah et al., 2005).

Oxidation of fatty acids is not only regulated by accessibility of substrates and the amount of product, but is also regulated at the level of gene expression. Transcription factors belonging to nuclear hormone receptor superfamily, namely peroxisome proliferators-activator receptors (PPARs) are known to up-regulate several proteins involved in fatty acid metabolism (Martin et al., 1997; Kramer et al., 2003; Mandard et al., 2004; Luci et al., 2006). A hypolipidemic drug—clofibrate, an activator of PPARα, was shown to increase carnitine concentration in rat liver (Paul and Adibi, 1979). Moreover, treatment of hepatoma cells with other PPARα activator–2-[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]acetic acid (WY-14,643) resulted in about twofold increase in mRNA coding OCTN2 (Luci et al., 2006). In the brain fibrates were observed to have neuroprotective effects in experimental model of Parkinson disease (Kreisler et al., 2007) and in ischemia (Bordet et al., 2006), which might imply significance of astrocyte peroxisomal metabolism on normal brain function. Presence of OCTN transporters was detected in various regions of mouse brain, including olfactory bulb, hippocampus, hypothalamus, cerebellum and motor cortex (Lamhonwah et al., 2008).

Since nothing has been known about the presence and activity of OCTN3 in astrocytes, this study was aimed at a detailed analysis of OCTN3 expression and localization, as well as a possible effect of PPARα activator on regulation of high affinity carnitine transporters in these cells.

2. Materials and methods

2.1. Materials

L-[Methyl-3H]carnitine, inulin[^{14}C]carboxylic acid and ECL kit were purchased from Amersham (Piscataway, NJ, USA). RNeasy Mini Kit and Mini Elute gel Extraction Kit were from Qiagen (Hamburg, Germany), DMEM (Dulbecco's modified Eagle medium) was from Life Technologies—Invitrogen (Carlsbad, CA, USA). Monoclonal antibody specific to glial fibrillary acidic protein—GFAP was from Chemicon International—Millipore (Bedford, MA, USA). Alexa Fluor 568 labeled anti-rabbit antibody, anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate and TO-PRO®-3 were from Molecular Probes—Invitrogen (Carlsbad, CA, USA). FITC-conjugated streptavidin and tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated streptavidin were from Jackson ImmunoResearch Europe Ltd. (Suffolk, UK). Dako® was from Dako (San Francisco, CA, USA). All other reagents were from Sigma (Poznań, Poland). The antipeptide polyclonal antibodies were obtained for OCTN proteins, for rOCTN2 the sequence 537–553 (SQTRTQKDGGESPTVLK, GI:5852403) was selected according to (Rodriguez et al., 2002). In case of rOCTN3 the peptide 525–540 (DEMQKVKRIRRVSAMS, GI:109488100; GI:109490711) was coupled with keyhole limpet hemocyanin (KLH) and the antibody was raised and affinity-purified by GenScript Corporation (Piscataway, NJ, USA).

2.2. Animals and pharmacological treatments

Eight adult Wistar albino rats were used in this study. Animals were housed individually in standard cages under 12/12 h light–dark cycle. All efforts were made to minimize the number of animals. The experimental procedures complied with the Polish Law on Experimentation on Animals that implements the European Council Directive of 24 November 1986 (86/609/EEC) and also with the NIH Guide for the Care and Use of Laboratory Animals. The experiments were approved and controlled by a Local Ethics Committee in Warsaw.

Rats were fed daily with restricted amount (18 g) of commercial standard diet, water was available *ad libitum*. Rats were divided into two groups each composed of 4 animals both sexes (2 males, 2 females). The first group of rats was treated by gavage with clofibrate (250 mg/kg in 1 ml of sunflower oil) for 4 days, whereas the second group was given sunflower oil (1 ml) to serve as control, according to a protocol described by Luci et al. (2006).

2.3. Tissue processing

After 4 days of treatment all animals were killed under deep anesthesia (intraperitoneal injections of 100 mg/kg of vetbutal). All animals were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion brains were removed, postfixed for 2 h in the perfusion solution and then immersed in 30% sucrose for 2 days before sectioning into 40 μm sections on a cryostat.

2.4. Cell culture

The 3-day old Wistar albino rats were sacrificed by decapitation according to the procedure approved by the Local Ethics Committee in Warsaw. Astrocytes were isolated as described by Booher and Sensenbrenner (1972) and cultured in 10% fetal bovine serum inactivated at 56 °C, 90% DMEM, supplemented with 2 mM L-glutamine and gentamycin (100 μg/ml) at 37 °C in a humid atmosphere of 5%