European Journal of Pharmacology 597 (2008) 51–56

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Behavioural Pharmacology

Modulatory effect of acetyl-L-carnitine on amyloid precursor protein metabolism in hippocampal neurons

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ARTICLE INFO

Article history:
Received 20 May 2008
Received in revised form 21 August 2008
Accepted 1 September 2008
Available online 6 September 2008

Keywords:
Alzheimer's disease
Acetylcarnitine
ADAM10
Amyloid precursor protein

ABSTRACT

Alzheimer Disease is the most common chronic neurodegenerative disorder associated with aging. Nevertheless, its pharmacological therapy is still an unresolved issue. In double-blind controlled studies, acetyl-L-carnitine (ALC) demonstrated beneficial effects on Alzheimer's disease. However, the mechanisms behind its neuroprotective ability remain to be fully established. In this study, the effect of acetyl-L-carnitine on amyloid precursor protein (APP) metabolism was investigated by in vitro models, both in a neuroblastoma cell line and in primary hippocampal cultures. We found that ALC treatment stimulates α-secretase activity and physiological APP metabolism. In particular, ALC favors the delivery of ADAM10 (a disintegrin and metalloproteinase 10, the most accredited candidate for α-secretase) to the post-synaptic compartment, and consequently positively modulates its enzymatic activity towards APP. Our findings suggest that the benefits of ALC reported in previous clinical studies are underscored by the specific biological mechanism of this compound on APP metabolism. In fact, ALC can directly influence the primary event in Alzheimer's disease pathogenesis, i.e. the Amyloid β cascade, promoting α-secretase activity and directly affecting the release of the non amyloidogenic metabolite.

1. Introduction

Nowadays, effective treatment is available for many neurologic conditions, but pharmacological therapy for dementia and, in particular, for Alzheimer's disease is still an unresolved issue. Significant improvements in cognition and global function were observed after treatment with cholinesterase inhibitors (Doody, 1999; Farlow, 2002; Rogers and Friedhoff, 1996), which were able to substantially control disease symptoms and to some extent to impinge on molecular pathogenic pathways (Eagger and Harvey, 1995; Farlow, 2002; Zimmermann et al., 2004, 2005). Indeed, basic research is clarifying many of the pathogenetic pathways that contribute to this devastating disease, providing unprecedented opportunities for the development of new treatments aimed at the root causes of Alzheimer's disease. According to the amyloid hypothesis, this disease is thought to be caused by the progressive accumulation and deposition of neurotoxic Amyloid β-peptide (Aβ) in amyloid plaques and aggregates in the brain (Selkoe, 2001). The production of Aβ is mediated by the concerted action of two different secretases, namely β-secretase (β-site APP Cleaving Enzyme, BACE) and γ-secretase, showing a proteolytic action on the amyloid precursor protein (APP). BACE cleaves APP at the N-terminus of the Aβ sequence, releasing into the extracellular space a soluble fragment, sAPPβ, and leaving attached to the extracellular membrane a 99 amminoacids long C-terminal fragment (CTF99). CTF99 can then be cleaved by γ-secretase exactly at the C-terminus of the Aβ sequence: this processing gives rise to the release of the amiloidogenic 4KDa Aβ fragment. On the other hand, the main protagonist of the physiological APP metabolic pathway is α-secretase, which cleaves APP within the sequence corresponding to Aβ thus preventing its formation (Haass, 2004). The most accredited candidate for α-secretase is a transmembrane metalloprotease, ADAM10 (a disintegrin and metalloproteinase 10) (Lammich et al., 1999; Postina et al., 2004). ADAM10 mediated non amiloidogenic pathway on APP releases one soluble, neurotrophic fragment called sAPPα and one membrane associated stub, called CTF83, which can then be cleaved by the γ-secretase complex. In particular, ADAM10 is active on APP when they both colocalize at the plasmatic membrane or along the secretory pathway (Lammich et al., 1999). Therefore the intracellular ADAM10 transport could be considered a possible key process in shifting APP processing toward the non-amyloidogenic pathway. It was recently reported that ADAM10 trafficking towards the membrane is mediated by SAP97 (Marcello et al., 2007), a cargo protein of the MAGUK (Membrane

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doi:10.1016/j.ejphar.2008.09.001

Associated Guanylate Kinase) family, known to be involved in dynamic trafficking of proteins to the excitatory synapse. Moreover, ADAM10 delivery to the postsynaptic density is primed by NMDA receptor activation and is mandatory for ADAM10 activity.

These primary mechanisms of pathogenesis are accompanied by a multiplicity of other pathogenic events concomitant with disease progression, i.e. oxidative stress (Smith et al., 1996) and impaired mitochondrial function (Lustbader et al., 2004). Overall, these mechanisms offer direct and/or indirect targets for therapeutic intervention.

Acetyl-L-carnitine (ALC) is an endogenous mitochondrial membrane compound that helps to maintain mitochondrial bioenergetics and lowers the increased oxidative stress associated with aging (Abdul et al., 2006). ALC is present in high concentrations in the brain and contains carnitine and acetyl moieties, both of which have neurobiological properties. Carnitine is important in the β-oxidation of fatty acids and the acetyl moiety can be used to maintain acetyl-CoA levels. Other reported neurobiological effects of ALC include modulation of brain energy and phospholipid metabolism, cellular macromolecules, including neurotrophic factors and neurohormones, synaptic morphology and synaptic transmission of multiple neurotransmitters (Pettegrew et al., 2000). In particular, ALC is active in cholinergic neurons, where it is involved in the production of acetylcholine (Dolezal and Tucek, 1981), membrane stabilization and enhancement of mitochondrial function (Hagen et al., 1998; Paradies et al., 1999). Given the involvement of the cholinergic system in Alzheimer's disease, ALC could play a protective role against Aβ mediated oxidative stress and neurotoxicity linked to the disease.

In double-blind controlled studies, ALC showed beneficial effects on Alzheimer's disease. Three placebo-controlled parallel design studies were reported, each studying the possible effect of ALC vs placebo in preventing the deterioration associated with this disease over a period of one year. On the whole, these studies suggest that ALC could indeed have a therapeutic effect in decreasing such deterioration. ALC treatment may be more beneficial in presenile than in senile stages of the disease (Spagnoli et al., 1991; Pettegrew et al., 1995; Thal et al., 1996).

Even if clinical studies on the effects of ALC are ongoing, the possible mechanism of action of ALC in Alzheimer's disease is still unclear. At present, it is known that pre-treatment of primary cortical neuronal cultures with ALC significantly attenuates Aβ induced cytotoxicity, protein oxidation and lipid peroxidation in a dose dependent manner (Abdul et al., 2006). Moreover, dietary administration of ALC for four weeks to Tg2576 mice leads to a significant decrease of Abeta 40/42 production compared to littermate-controls or wild-type mice (Chauhan and Siegel, 2003). Thus, the aim of this study is to determine whether ALC treatment may affect primary pathogenic mechanisms of Alzheimer's disease, i.e. the amyloid cascade, in in vitro systems.

2. Materials and methods

2.1. Cell culture and pharmacological treatment

Cell culture was performed as described (Lahiri et al., 1998) with minor modifications. Human neuroblastoma cells (SH-SY5Y) were used because they are a representative cell model of a human cell line and can be differentiated to a cholinergic phenotype (Zimmermann et al., 2004). SH-SY5Y were cultured in minimum Eagle's medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Celbio, Pero, Italy), 1% L-glutamine (Sigma-Aldrich) and 1% penicillin–streptomycin (Sigma-Aldrich). Cells were seeded on 35 mm tissue culture wells (250,000 cells per wells) and 24 h afterwards, all-trans-retinoic acid 5 μM (Sigma-Aldrich) was added. This treatment was repeated every two days for six days to achieve cell differentiation (Bartolini et al., 2003). Before adding the drug, cells were starved for twelve h in MEM not containing FBS. Cells were treated in the presence of ALC (Sigma-Aldrich); different concentrations of ALC, ranging from 150 μM to 300 μM for either 2 or 4 h, were used to find the optimal concentration. Phorbol dibutyrate, PBDu (Sigma-Aldrich), was added at 0.1 μM for 15 min, because it is an activator of PKC, which has been shown to increase α-secretase cleavage of APP (Desdouits et al., 1996). Unless indicated otherwise, $N=3$ means that three independent assays were performed on three plates containing the same number of cells for each treatment. Cell morphology was monitored before and after differentiation and treatment. Differentiation manifested itself in an arrest of growth after six days of cell treatment (assessed by phase-contrast microscopy with tryptan blue exclusion method). Additionally, cells underwent dissociation of cell aggregates after treatment with retinoic acid. Differentiation was considered completed as described elsewhere by considering the length of growth cone-terminated neurites vs cell body diameter (Poongodi et al., 2002; Kraveka et al., 2003): the growth cone-terminated neurites of differentiated cells appeared to be up to three times longer than the diameter of the corresponding cell body as compared to undifferentiated cells where the neurites were shorter than the diameter of the corresponding cell body.

2.2. Hippocampal neuronal cultures and treatment

Hippocampal neuronal cultures were prepared from E19 rat hippocampi as described (Gardoni et al., 2002) and used for treatment with ALC at 21 days in vitro (DIV). Neurons were treated in the presence of 150 μM ALC for 2 and 4 h. All experimental procedures were carried on with care to minimize discomfort and pain to treated animals, in accordance with the guidelines of the European Communities Council (Directive of November 24, 1986, 86/609/EEC).

2.3. Triton-X Insoluble Fraction preparation

Triton-X100 Insoluble Fractions from hippocampal neuronal cultures were purified as previously described (Gardoni et al., 2001).

2.4. Protein evaluation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

To quantify sAPPα, the SH-SY5Y neuroblastoma media were desalted by using DG10 columns (Bio-RAD, CA, USA), lyophilized and resuspended in Laemmli's buffer. The total proteins were separated on 6% SDS-PAGE and electroblotted onto nitrocellulose membranes. For the evaluation of ADAM10, SAP97, CTF99, CTF83, Tubulin and APP in cell homogenates and subcellular fractions, equal amounts of total proteins were loaded per lane, while evaluation of sAPPα was achieved by loading the total lyophilizate obtained from the medium of one plate onto SDS-PAGE and normalizing OD (optical density) to the total proteins per corresponding cell well. Acetylcholinesterase's activity in cell lysates was determined as previously described (Zimmermann et al., 2004). Cell proteins were separated in 6% SDS-PAGE for APP and 7% for SAP97, Tubulin and ADAM10, respectively, and Western blot analyses performed as previously described (Di Luca et al., 1996). Triton-X Insoluble Fraction of rat primary cell cultures was separated in 15% Tris–Tricine for CTF99 and CTF83 analysis. Immunostaining reactions were performed using monoclonal antibody (mAb) 22C11, raised against APP N-Terminal domain, for APP (Chemicon International, Inc., Tamecula, CA, USA; dilution 1:2500), mAb 6E10 (Chemicon International, Inc.; dilution 1:3000) for sAPPα, mAb 4G8, raised against 17–24 aa of Aβ, for CTFs (Chemicon International, Inc., Tamecula, CA, USA; dilution 1:2500), mAb against SAP97 (Stressgen Biotechnologies, Victoria, Canada; dilution 1:1000), polyclonal antibody (pAbs) anti-ADAM10 N-terminal (Sigma, St. Louis, MO, USA; dilution 1:3000), and mAb anti-Tubulin (Sigma-Aldrich). After incubation with peroxidase conjugated secondary antibodies (Pierce, Rockford, IL, USA; dilution 1:10000), blots were developed with enhanced chemiluminescence (Amersham-Pharmacia Biotech, Little Chalfont, UK).