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Activation of α-secretase by curcumin-aminoacid conjugates

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ABSTRACT

The extracellular senile plaques observed in Alzheimer's disease (AD) patients are mainly composed of amyloid peptides produced from the β-amyloid precursor protein (βAPP) by β- and γ-secretases. A third non-amyloidogenic α-secretase activity performed by the disintegrins ADAM10 and ADAM17 occurs in the middle of the amyloid-β peptide Aβ and liberates the large sAPPα neuroprotective fragment. Since the activation of α-secretase recently emerged as a promising therapeutic approach to treat AD, the identification of natural compounds able to trigger this cleavage is highly required. Here we describe new curcumin-based modified compounds as α-secretase activators. We established that the aminoacid conjugates curcumin–isoleucine, curcumin–phenylalanine and curcumin–valine promote the constitutive α-secretase activity and increase ADAM10 immunoreactivity. Strickingly, experiments carried out under conditions mimicking the PKC/muscarinic receptor-regulated pathway display different patterns of activation by these compounds. Altogether, our data identified new lead natural compounds for the future development of powerful and stable α-secretase activators and established that some of these molecules are able to discriminate between the constitutive and regulated α-secretase pathways.

1. Introduction

The main component of the senile plaques observed in AD patients is a 40- to 43-amino-acid-long β-amyloid (Aβ) peptide that is produced from the β-amyloid precursor protein (βAPP) through sequential cleavages by β- and γ-secretase [1]. Thus, intense efforts have focused on the development of specific and powerful inhibitors of these activities. However, the fact that both enzymes cleave numerous other physiologically relevant substrates renders their inhibition problematic because of deleterious secondary effects on the nervous system, the immune system and the gastrointestinal tract [2–5]. Therefore, a promising but yet underestimated strategy would be to activate the α-secretase processing of βAPP [6]. This cleavage is performed by the disintegrins ADAM10 and ADAM17 that are responsible for the constitutive and PKC-regulated pathways, respectively [7,8]. Interestingly, this cleavage can be seen as twice beneficial since it both occurs in the middle of the Aβ sequence and gives rise to the production of the neurotrophic and neuroprotective secreted sAPPα fragment [6]. In addition to protein kinase C (PKC) for which deficits were observed in Alzheimer's disease patients [9], M1/M3 subclass of muscarinic receptors have been shown to be key up-regulators of α-secretase processing of βAPP *in vitro* and *in vivo* [10,11].

Chronic or acute pharmacological stimulations of α-secretase would engender many deleterious consequences, especially when one considers the tumor-promoting activity of the PKC-activating phorbol esters. The demonstration that the natural compound bryostatin is able, at sub-nanomolar concentrations, to trigger PKC activity, promote sAPPα secretion and reduce Aβ production without inducing tumor represents a step forward in the search of potent and harmless PKC activators [12]. The alternative solution consisting in the activation of α-secretases themselves, would face a major issue since ADAM10 and ADAM17 target more than 80 different substrates, some of which being implicated in the development of severe pathologies [13]. Thus, it may appear more judicious to privilegiate the use of long-term and mild treatments, mainly through the consumption of natural compounds present in food. In this context it recently emerged that several natural compounds such as the plant extracts *Ginkgo biloba* and green tea–epigallocatechin-3-gallate can specifically activate the α-secretase cleavage of βAPP [14–15].

Curcumin (diferuloylmethane), the main component extracted from the plant *Curcuma longa* (turmeric), can penetrate the

Abbreviations: PKC, protein kinase C; ADAM, A disintegrin and metalloprotease; PDBu, phorbol-12,13-dibutyrate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; HEK, human embryonic kidney.

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blood–brain barrier and was suggested as a promising therapy for AD since it displays anti-inflammatory, antioxidant and copper and iron chelation properties [16]. A role for curcumin in the regulation of βAPP biology and processing emerged during the past 3 years when curcumin was shown to impair Aβ42 production, to reduce βAPP protein levels, to suppress Aβ-induced BACE1 upregulation and to attenuate βAPP maturation in the secretory pathway, thereby decreasing Aβ levels [17,18]. However, the influence of curcumin on the α-secretase cleavage of βAPP has not been investigated so far.

Because of its low absorption, rapid metabolism, inherent instability and hydrophobic nature, clinical trials carried out with curcumin did not show any improvement of cognitive functions in humans with dementia [19]. Therefore, efforts have been made to increase its bioavailability and the use of adjuvants such as piperine ameliorates curcumin stability although no significant improvements were evidenced in mild-to-moderate AD patients [20]. This suggests that further chemical modifications of curcumin are needed to trigger some beneficial effects. In the present study, we examined the effects of curcumin, its metabolite tetrahydrocurcumin (THC) as well as the three aminoacid conjugates curcumin–isoleucine, curcumin–phenylalanine and curcumin–valine on the non-amyloidogenic α-secretase activity. We report that the aminoacid conjugates induce the constitutive α-secretase cleavage of both the synthetic substrate JMV2770 and βAPP through an increase in ADAM10 immunoreactivity. Moreover, it appears that these compounds are able to discriminate between the constitutive and the regulated α-secretase pathways.

2. Materials and methods

2.1. Antibodies, reagents and cell lines

Polyclonal anti-ADAM10 and anti-ADAM17 were from Millipore. Polyclonal anti-βAPP (A8717) was from Sigma. Monoclonal anti-β-amyloid (DE2B4) which was used to specifically detect sAPPα and monoclonal anti-actin were from Cell Signaling. DMEM complete medium and fetal bovine serum were from Gibthai-Invitrogen Corporation. Penicillin–streptomycin mix was from PAA. Tris buffer and Glycine were from Vivants. Dimethyl sulfoxide and sodium bicarbonate were from Sigma. Skim milk powder was from Criterion. ECL and ammonium persulphate were from GE Health care. SDS was from Amaresco. Metalloprotease inhibitor *o*-phenanthroline was from CalBiochem. The α-secretase fluorimetric substrate JMV2770 is an 11-amino-acid-long sequence of the cellular prion protein encompassing the peptidyl bond targeted by α-secretase. JMV2770 (Abz-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Gln-EDDnp) contains the ortho-aminobenzoyl (Abz)/dinitro-phenyl (EDDnp) groups as the donor/acceptor and was synthetized as previously described [21]. HEK 293 cells overexpressing ADAM10, ADAM17, M1R and βAPP were cultured as previously described [22–25].

2.2. Curcumin and curcumin derivatives

Curcumin (~95% purity) was purchased from Ms Spicex chemicals Pvt. ltd. (Mysore, India). The aminoacid conjugates curcumin–isoleucine (Cur–Ile), curcumin–phenylalanine (Cur–Phe) and curcumin–valine (Cur–Val) were prepared on gram scale using the previously described protocol [26] (see Fig. 1 for the detailed structures of the compounds).

2.3. Fluorimetric assay on intact cells

HEK293 cells overexpressing ADAM10, ADAM17 or M1R were cultured in 35-mm dishes until cells reach 80% confluency. Cells were treated in duplicate without (control) or with 50 μM curcumin, THC, Cur–Ile, Cur–Phe and Cur–Val for 14 h at 37 °C in DMEM containing 1% FBS. After this treatment period, the PKC activator phorbol-12,13-dibutyrate (PDBu) (1 μM, ADAM17-overexpressing cells) or the muscarinic agonist carbachol (100 μM, M1R-overexpressing cells) were added for 2 h. Duplicates were then incubated for 30 min at 37 °C either with or without *o*-phenanthroline (100 μM in 1.5 ml of PBS). Then, JMV2770 substrate (10 μM) was directly added into the media and cells were maintained at 37 °C. After 10, 30 min and subsequently at every 30 min, 100 μl of media were removed and fluorescence was recorded in black 96-well plates at 320 and 420 nm excitation and emission wavelengths, respectively. After the last incubation time, cells were resuspended in 10 mM Tris, pH 7.4 and samples were kept for Western blot analysis.

2.4. Western blot analyses

Ten micrograms of proteins were loaded onto 8% (ADAM10 and ADAM17) or 10% (β-actin) SDS–PAGE and run at 120 V for 90 min and then transferred onto nitrocellulose membrane for 90 min at 100 V. Patterns of transferred proteins were checked with Ponceau Red staining and nitrocelluloses were incubated in 5% nonfat milk blocking solution for 30 min. Membranes were then incubated with primary antibodies directed against ADAM10 (1/1000 dilution), ADAM17 (1/1000) or β-actin (1/5000) on a platform shaker overnight at 4 °C. After three washes with PBST (PBS containing 0.05% Tween 20), membranes were incubated with HRP-conjugated anti-rabbit (ADAM10 and ADAM17) or anti-mouse (β-actin) secondary antibodies (1/5000) for 2 h and rinsed three times with PBST. Immunoreactivities were processed using ECL and signals were detected using an automatic X-ray developing machine. Films were then analyzed by densitometry and ADAM10 and ADAM17 levels were normalized using β-actin as an internal standard.

2.5. Effect of curcumin and its conjugates on βAPP processing

HEK293 overexpressing βAPP were cultured in 35-mm dishes until cells reach 80% confluency. Cells were treated without (control) or with 50 μM curcumin and its conjugates THC, Cur–Ile, Cur–Phe and Cur–Val for 14 h at 37 °C in DMEM containing 1% FBS. Media were then removed and replaced with DMEM without FBS and cells were allowed to secrete for 1 h. Both media (20 μl, sAPPα) and cell lysates (20 μg of proteins for βAPP; 10 μg of proteins for β-actin) were submitted to Western blot analysis. Samples were loaded onto 8% (sAPPα and βAPP) or 10% (β-actin) SDS–PAGE, run at 120 V for 90 min and transferred onto nitrocellulose membrane for 120 min at 100 V. Nitrocelluloses were incubated in 5% nonfat milk blocking solution for 30 min and incubated with primary antibodies directed against sAPPα (1/500 dilution), βAPP (1/5000) or β-actin (1/500) overnight at 4 °C. After three washes with PBST, membranes were incubated with HRP-conjugated anti-rabbit (βAPP) or anti-mouse (sAPPα and β-actin) antibodies for 2 h and rinsed three times with PBST. Immunoreactivities were processed using ECL and signals were detected using an automatic X-ray developing machine. Films were then analyzed by densitometry and βAPP levels were normalized using β-actin as an internal standard.

2.6. Statistical analysis

Statistical analyses were performed with the Prism software (Graphpad, San Diego, USA) by using the Newman–Keuls multiple comparison test for one-way ANOVA and the Student's *t* test for pairwise comparisons. All the results are expressed as means ± SEM values.