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OVEREXPRESSION OF CLATHRIN IN HIPPOCAMPUS OF ALZHEIMER'S DISEASE RAT TREATED WITH LAVANDULA ANGUSTIFOLIA

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ABSTRACT: Endocytosis plays a central role in the production of amyloid-β (Aβ) in neurons. Clathrin and proteins regulate clathrin-mediated endocytosis are dysregulated in Alzheimer's disease (AD) which might cause the enlargement of axon terminals and lead in impaired neurotrophic factor signaling that involves clathrin-mediated endocytosis of activated receptors. Lavandula angustifolia (LA) previously specified to improve the spatial performance in AD animal model by reduction in Aβ production in histopathology of hippocampus, also determined linalool produce marked antinociception against glutamate induced pain in mice, possible due mechanisms operated by ionotropic glutamate receptors, AMPA, N-methyl-D-aspartate (NMDA)and kainite, so in this study neuroprotective mechanisems of lavender extract (LE) in Aβ injected rat was investigated by proteomics techniques with emphasis on clathrin expression. Rats were allocated to three groups included normal (N); Aβ injected (Aβ) and Aβ injected rat and treated with lavender aqueous extract (Aβ□□LE). Hippocampus tissues of three groups were separated and protein profile was determined by 2DE and proteins identified by MALDI-TOF/TOF. By searching deeply in DAVID Bioinformatics Resources, clathrin was matched to specific processes or functions and used STRING online database to determine predicted interactions of clathrin with other proteins. Result illustrated that clathrin expressed for 6 fold more in Aβ□□LE group than others and binds directly to proteins such as Cltc, Epn1, Cltb, Ap2m1, Dnm3, RT1-Da, Dnm2, Egfr, Ap2b1 and Egf which activated in endocytosis dependent clathrin. Some of these proteins are presented in pathogenesis of other neurodegenerative diseases. So neuroprotective action of clathrin might be consequently associated to elevate clathrin-mediated NMDAR endocytosis followed by increased intracellular α-Synuclein (α-syn) levels that exhibited in present of lavender, reduce surface NR1 without altering the total NR1. Because the α-syn-induced surface NR1 reduction was accompanied with suppression of NMDA-elicited intracellular Ca2+ elevation and reductions of NMDA-induced caspase 3 activation and cell death. In sum up highly level expression of clathrin in presence of LA component related to increase endocytosis thereby uptakes nutrients and effective drug agents into damaged cells likewise decreased glutamate receptors in cell surface.

Abbreviation: Alzheimer's disease (AD), Amyloid-β(Aβ), Lavandula Angustifolia (LA), Lavender Extract (LE), Gene Ontology (GO), Huntington's Disease (HD), N-methyl-D-aspartate (NMDA), α-Synuclein (α-syn), clathrin light chain A (CLCA), clathrin A (Clta), Huntingtin Interacting Protein (HIP1), Huntingtin's Interaction Protein 1 interactor (HIPPI)

Keywords: Alzheimer's Disease, Lavandula Angustifolia, Proteomics, Hippocampus, Clathrin.

INTRODUCTION

Clathrin protein, part of coated vesicles, act as molecular transport machine within and between cells. Clathrin-mediated endocytosis and exocytosis deal with transferring nutrients, importing signaling receptors, mediating an immune response, cleaning up the cell debris left by tissue inflammation, recycling of plasma membrane

components, and destination surface proteins for degradation (1, 2). Clathrin-mediated endocytosis in nerve terminals regulates synaptic transmission (3) and is crucial for synaptic vesicle recycling (2). One important aspect in the neuropathology of AD is irregular synaptic vesicle trafficking which lead in of synaptic losses and contributes to the progressive cognitive impairment in AD. Aberrant in expression of proteins related to synaptic vesicle trafficking could have direct effect on the functionality of neuronal circuits (4). Process of synaptic vesicle recycling may also be abnormal in AD (5) so that the number of proteins such as synaptotagmin, dynamin, AP2 and AP1 that regulate clathrin-mediated vesicle recycling are reduced in AD (6, 7). The amyloidogenic processing by β -secretase/□-secretase takes place in endosomes (8-10), the production of Aβ is dependent on the endocytosis of APP from the cell surface and its transit to the endosomes. Indeed, cells expressing endocytosis deficient APP produce significantly less Aβ (11). Numb may serve as a modulator of APP sorting by interaction directly to intracellular part of APP and promotes APP travel towards the endosomes where APP can be processed by β secretase and □-secretase to produce Aβ. α secretase predominantly localizes to the cellular membrane whereas β secretase to acidic intracellular compartments (endosomes) (12-14) to generate and secrete Aβ peptide to the interstitial fluid (15). Aβ have opposed effects on the pre-and post-synaptic functions and integrity (16) which one result may be abnormalities in axons due to the effects of Aβ on tau and microtubules consequently lead in neurofibrillary tangle formation and cell death (17). Disturbance in synaptic vesicle trafficking in presynaptic terminals and axonal transport may cause dysfunction and neuron death in AD. Enhancing activation of such synapses by drugs like acetylcholinesterase inhibitors, can recuperate cognition during the early stages of disease (18).

Today's used natural product of plants extensively in dementia therapy and memory enhancers. Among common components in plants terpenoids with low molecular weight and high hydrophobicity have good chance to cross cellular membranes and the blood–brain barrier and effect on brain regions (19). Throughout history LA is a plant which belongs to the 'Labiatae' family that used for multiple pharmacological effects such as anticonvulsant, sedative, antispasmodic, analgesic, antioxidant and local anaesthetic activity (20-23). Soheili Kashani M et.al beforehand demonstrated that LE eliminates Aβ plaques in the hippocampus of AD animal model (24), therefor; reverse effectively spatial learning deficits in AD rats (25). Experimental evidences indicate that beneficial effects of linalool of LA produce marked antinociception against glutamate induced pain in mice, possible due mechanisms operated by ionotropic glutamate receptors, namely AMPA, NMDA and kainite(26). So in this study, the mechanism(s) of the neuroprotective effects of LA extract on clear Aβ plaques from rat hippocampus was investigated based on proteomics approach with emphasis on clathrin expression.

MATERIALS AND METHODS

Animals

Adult male wistar rats, weighing 250–300 g were housed three to four per cage in a temperature-controlled colony room under light/dark cycle and free access to water and food throughout the experiment. This study was conducted in accordance with the policies stipulated in the Guide for the Care and Use of Laboratory Animals (NIH).

Experimental Procedure

There exist 3 rat groups: normal (N; n = 10); Aβ injected (Aβ; n = 10) and Aβ injected and treated with lavender (Aβ□□□LE; n = 10). Stereotaxic surgery was done according to the stereotaxic atlas (27) and Aβ 1–40 (Sigma Aldrich, St. Louis, MO, USA) was injected at coordinates of –3.5 mm posterior to bregma, 2 mm lateral to sagittal suture, and 2.8 mm below dura. The animals in control group were treated with the same procedure except that they received distilled water.

Lavender aqueous extract prepare according to Soheili procedure (25). 20 days after establishing AD model, lavender extract (200 mg/kg) administrated as intraperitoneally injected once per day for 20 consecutive days. The dosage was chosen according to the results of our pilot study and an earlier investigation (25). The normal groups were either injected distilled water.

Sample Preparation and Two Dimensional Gel Electrophoresis (2DE)

Fresh hippocampus tissues were snap frozen and kept in liquid nitrogen until used. Hippocampus were washed then homogenized by pestle in lysis buffer. All stage of sample preparation and two dimensional gel electrophoresis were performed according to our previous study (28). Each sample was loaded onto 11 cm immobilized (Ph=3-10) nonlinear gradient strips (Bio-Rad, Hercules, CA, USA). The IPG strips were placed on 12% polyacrylamide gels and resulting gels were stained with Coomassie Brilliant Blue (29).

Protein Identification by MALDI-TOF/TOF

In-gel protein digestion was performed according to Zhou et al. with minor modifications (30). The data search was conducted on GPS Explorer (Version 3.6, AB SCIEX) using the search engine Mascot (Version 2.2, Matrix Science, London, UK), and the International Protein Index (IPI) rat database (vision 3.64, 39871sequences, http://www.ebi.ac.uk/IPI) was used for peptide and protein identification. General protein identification was based on two or more peptides whose ion scores surpassed the statistical threshold ($p<0.05$).

Bioinformatical and Statistical Analysis

Scanned 2DE gels are analyzed by nonlinear progenesis same spot software to compare gels together and compare the spots in one statement in gels and get the density of same spot in each of gel. To detect significant differences between the experimental groups, analysis of variance (ANOVAs) were used. A p-value <0.05 was considered to be statistically significant. Statistics were presented as means±SE.

The identified proteins were then matched to specific processes or functions by searching the Gene Ontology (GO), INTERPRO, KEGG_PATHWAY, PIR_SUPERFAMILY, SP_PIR_KEYWORDS, UP_SEQ_FEATURE and UP_TISSUE in DAVID Bioinformatics Resources 6.7 (the Database for Annotation, Visualization, and Integrated Discovery) "http://david.abcc.ncifcrf.gov/" (31).

To determine predicted interactions of clathrin with other proteins as functional protein association network was obtained by searching the STRING online database (http://string-db.org).

RESULTS

To explore the molecular mechanism underlying the beneficial effect of lavender aqueous extract on neuron plasma membrane of AD rat hippocampus, 2DE-based proteomics was utilized in the N, Aβ and Aβ + LE groups. As shown in Fig. 1, clathrin spot were detected by Coomassie Brilliant Blue in three 2DE maps then validated by MALDI-TOF/TOF analysis and represented different expression in three groups. By differential analysis with nonlinear progenesis same spot software, determined expression 2506.262 in N, 4229.091 in Aβ and 14950 in Aβ + LE group. There are about 6 folds difference between the lowest and highest expression with significance P. value < 3.12E-07(Anova (p)). Clathrin was comprehensively explained in Table 1. By searching the STRING online database predicted interaction proteins with clathrin (Clta). This functional protein association network for the entry "Clta," binds directly to proteins such as Cltc, Epn1, Cltb, Ap2m1, Dnm3, RT1-Da, Dnm2, Egfr, Ap2b1 and Egf.

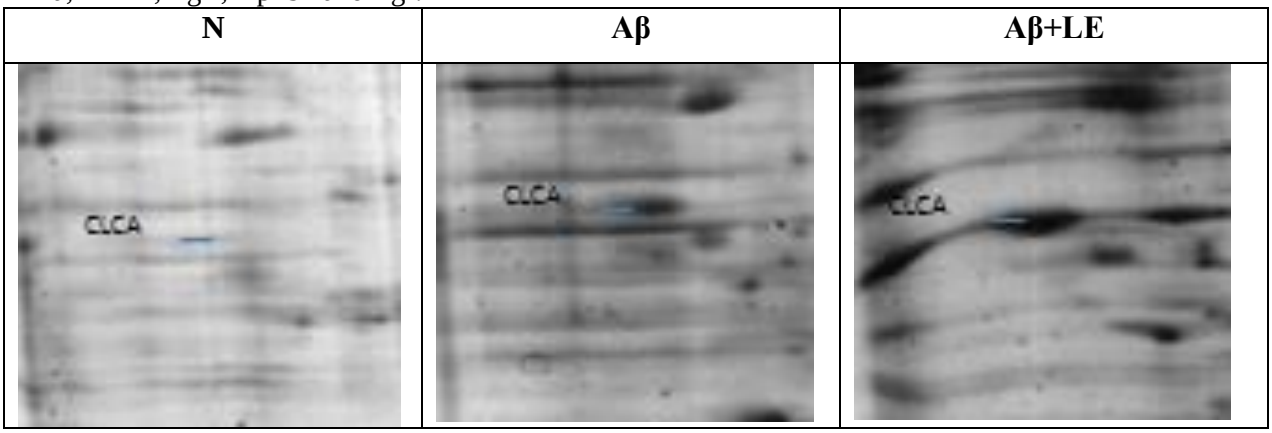

Fig. 1. Representative two-dimensional gel maps of clathrin that show differential levels in N, Aβ and Aβ +LE groups.

Table 1: Different categories of clathrin (P08081) analyzed based on some important databases in DAVID Bioinformatics Resources 6.7

P08081	Clathrin, light chain (Lca)
GOTERM_BP_FAT	Intracellular protein transport, post-Golgi vesicle-mediated transport, endocytosis, protein localization, membrane invagination,protein transport, membrane organization, vesicle-mediated transport, cellular protein localization, establishment of protein localization, intracellular transport, Golgi vesicle transport, cellular macromolecule

P08081	Clathrin, light chain (Lca)
	localization
GOTERM_CC_FAT	Golgi membrane, Golgi apparatus, Golgi-associated vesicle, plasma membrane, coated pit, internal side of plasma membrane,endomembrane system, vesicle membrane, trans-Golgi network transport vesicle membrane, cytoplasmic membrane-bounded vesicle, membrane coat, clathrin coat, vesicle coat, clathrin vesicle coat, clathrin coat of trans-Golgi network vesicle, clathrin coat of coated pit, transport vesicle, coated vesicle, clathrin-coated vesicle, endocytic vesicle, trans-Golgi network transport vesicle,transport vesicle membrane, cytoplasmic vesicle membrane, Golgi-associated vesicle membrane, coated vesicle membrane,clathrin coated vesicle membrane, endocytic vesicle membrane, clathrin-coated endocytic vesicle membrane, organelle membrane, cytoplasmic vesicle, vesicle, membrane-bounded vesicle, Golgi apparatus part, cytoplasmic vesicle part, plasma membrane part, clathrin-coated endocytic vesicle, coated membrane
GOTERM_MF_FAT	Structural molecule activity, calcium ion binding, peptide binding, ion binding, cation binding, metal ion binding
INTERPRO	Clathrin light chain
KEGG_PATHWAY	Lysosome, Endocytosis, Huntington's disease,
PIR_SUPERFAMILY	PIRSF002289:clathrin light chain,
SP_PIR_KEYWORDS	Alternative splicing, calcium, coated pit, coated pits, coiled coil, cytoplasmic vesicle, direct protein sequencing, Endocytosis,membrane, phosphoprotein,
UP_SEQ_FEATURE	Chain:Clathrin light chain A, modified residue, region of interest:Involved in binding clathrin heavy chain, splice variant,
UP_TISSUE	Hippocampus, Ovary,

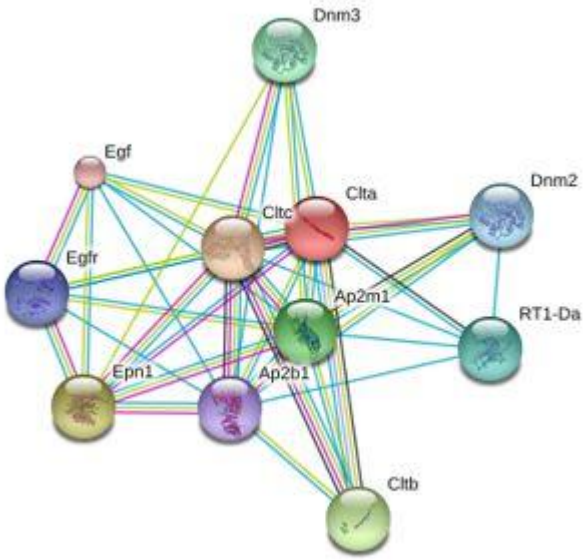

Fig3. Known and predicted interactions of Clta with other proteins. This functional protein association network was obtained by searching the STRING online database (http://string-db.org)

DISCUSSION

The hippocampus as a part of limbic system plays important roles in the unification of information from short-term memory to long-term memory and spatial navigation. Hippocampus in AD is one of the first regions suffer damage and memory loss (32). In AD animal, acute treatment with soluble oligomeric Aβ disrupts synaptic plasticity and causes inhibition of long-term potentiation and enhancement of long-term depression of glutamatergic transmission that finally implicates reducing synaptic integrity (33). Reactive astrocytes, activated microglia, chronic inflammation, excitotoxic damage accompanied by altered ion homeostasis, altered energy metabolism, oxidative stress and altered antioxidant defense systems (34). In addition to NMDA and possibly nicotinic receptors are mediating the disruptive effect of Aβ which targeting muscarinic receptors that able indirectly modulate Aβ actions (33). Impaired endocytosis is also detected with enlarged endosomes as an early neuropathological finding (35). Previously illustrated the protective effect of aqueous extract of lavender on eliminating Aβ in intrahippocampal Aβ-injected rat model of AD (24,25), so, the aim of the present study was to evaluate the mechanism of this beneficial effect by evaluating clathrin expression in three groups (N, Aβ and Aβ+LE) (Figure 2). Aqueous methanolic extracts of LA contains rosmarinic acid, caffeic acid, luteolin 7-Oglucoside, methyl carnosoate (36). Neurologic effects of LA fragrance stimuli increased arousal and relaxation based on positron emission tomography of various brain sections (37). Linalool has been determined to diminish motor activity in mice because of a dose-related binding to glutamate and also suggested that neurotransmitter GABA may be responsible hypnotic and anticonvulsant effects of lavender (38). The mechanism of LA beneficial effects on anxiety and mood was included interactions with NMDA or GABAA receptors, voltage dependent sodium channels, or glutamatergic and cholinergic neurotransmission (39-42). In this study clathrin light chain A (CLCA) identified in hippocampus was spotlighted. It is the major protein of the polyhedral coat of coated pits and vesicles have been linked to signal regulation and neural transmittance. Result depicted that the level of CLCA not only was found to increase in AD but also in presence of LE was up-regulated for 6 fold than normal. Aβ is cleaved from APP prevalently after APP exocytosis and then become re-internalized by clathrin-mediated and clathrin-independent endocytosis (43). Furthermore, significant increases in the levels of clathrin identified in AD transgenic animals, implicated in endocytic abnormalities (44) and abnormal distribution implied impairment of axonal transport in AD; moreover it play in the HD (45-48). Since clathrin-coated vesicle display in endocytosis, intracellular protein transport, protein localization, membrane invagination, intracellular transport, golgi vesicle transport, cellular macromolecule localization, highly express of clathrin in neurons in presence of LE may be control the uptake of lavender components, endocytosis surface receptor or brings needed nutrients into the damaged cell(46). However, receptor-mediated endocytosis as a result of polymerization of clathrin molecules on the cytoplasmic face of the plasma membrane is the major cellular route for uptake of macromolecular drugs and their metabolism in subcellular compartments in acidic conditions. The polymerized clathrin cause internalization cell surface receptors along with bound ligands (49-50). In addition to clathrin involved in bringing nutrients to the cell, binds to the Huntingtin Interacting Protein (HIP1) right next door to where Huntingtin's Interaction Protein 1 interactor (HIPPI) binds. While clathrin packages nutrients for a cell, HIP1 connects these baskets to the structure of the cell. If HIPPI binding with HIP1 prevents clathrin connection with HIP1, then the normal pathway of nutrients into a cell is interrupted, ultimately trigger the chain of interactions leading to HD (46). Beside on, clathrin interactome dysfunction may therefore be a risk factor for psychotic illness (51). In AD, clathrin-dependent endocytosis of APP is believed to be the rate-limiting step in the production of Aβ peptide whose accumulation is the hallmark of the disorder (43, 52–54). Further, clathrin

was found to interact with two proteins, Snca and DJ-1, critically involved in the pathogenesis of Parkinson's disease (55). These observations show that alterations of proteins even remotely linked to clathrin-dependent processes can lead to significant disturbances of the nervous system (44). It is intriguing to speculate that perturbance of the clathrin interactome increases the likelihood of developing psychosis as part of the phenotype in AD patients. In general according to STRING database clathrin bind directly to Cltc, Epn1, Cltb, Ap2m1, Dnm3, RT1-Da, Dnm2, Egfr, Ap2b1 and Egf that activated in endocytosis. Previously illustrated Clta, Cltc, Cltb, Ap2m1 and Ap2b1 are involved in HD pathogenesis. AP180 as adaptor molecules is used in synaptic vesicle formation. AP180 and Epsin recruit clathrin to membranes and also promote its polymerization. Epsin also can help deform the membrane, and thus clathrin-coated vesicles can bud (56-59). In AD, different factor like increasing cholesterol could be responsible for the enhanced internalization of clathrin, dynamin2, Eps15 and Rab5 dependent endocytosis of APP and the consequence overproduction of Aβ (60). As earlier explained, perturbations in systems using the excitatory amino acid L-glutamate may underlie the pathogenic mechanisms of AD. Since NMDA subtype of ionotropic L-glutamate receptors exist in the most neurons in the CNS and can mediate post-synaptic Ca2+ influx, so excessive activation of NMDA receptors by excitotoxicity could imitate neuron vulnerability in a manner seen in AD neuropathology. NMDA receptor antagonists or diminishing its expression have potential for the therapeutic improvement of AD(61). In addition to previously demonstrated abnormalities of α-syn and NMDA receptors involved in pathogenesis of Parkinson's Disease so that increased intracellular α-syn levels lead to reduced surface NR1 without altering the total NR1. The α-syn-induced surface NR1 reduction was accomplished with repression intracellular Ca2+ elevation and reductions of NMDA-induced caspase 3 activation and cell death. So, α-syn may promote clathrin-mediated NMDAR endocytosis (62). Thereby expression of α-syn in our study (not published yet) in Aβ+LE groups has a protective effect with overexpress of clathrin to increase clathrin-mediated NMDAR endocytosis. Since in this study, Aβ were injected in rat brain, highly expression of clathrin in Aβ+LE is not correlated to APP process, so might be related to cellular uptake of LA components to neurons principally via clathrin-mediated endocytosis or modulate glutamate-mediated excitotoxicity by clathrin-mediated NMDAR endocytosis consequently function with different combinations and permutations of regulators to meet the specific physiological demands of neuroprotective processes.

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