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Effects of curcumin on hippocampal expression of NgR and axonal regeneration in Aβ-induced cognitive disorder rats

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ABSTRACT. Curcumin has been widely used for the prevention and treatment of Alzheimer's disease (AD), but its mechanism is still not clear. Inhibitory factors of axonal regeneration have been shown to cause a series of pathophysiological changes in the early period of AD. In this study, the co-receptor (Nogo receptor; NgR) of three axonal growth-inhibitory proteins was examined, and effects of curcumin on spatial learning and memory abilities and hippocampal axonal growth were investigated in amyloid β-protein (Aβ)1-40-induced AD rats. Results showed that the expression of NgR in the AD group significantly increased and the number of axonal protein-positive fibers significantly reduced. The spatial learning and memory abilities of AD rats were significantly improved in the curcumin group. Furthermore, hippocampal expressions of NgR mRNA and protein decreased, and the expression of axonal protein significantly increased. There was a negative correlation between the expression of NgR and axonal growth. Together, these results suggested that curcumin could improve the spatial learning and memory abilities of AD rats. The mechanism

might be related with its lowering of hippocampal NgR expression and promoting axonal regeneration.

Key words: Curcumin; Amyloid β protein; NgR; Axon; Alzheimer's disease

INTRODUCTION

The *Curcuma* rhizome, belonging to the Zingiberaceae family, is the root of the *Curcuma longa* plant. *Curcuma* has a long history of use as traditional medicine in China and India, and it is used as a curry spice in food preparation (Wang and Du, 2003). Curcumin ($C_{21}H_{20}O_6$) is the main pharmacologically active ingredient of the *Curcuma* rhizome. It is safe, with little to no toxicity, and possesses antitumor, antioxidant, anti-inflammatory, anti-apoptosis, and lipid-reducing pharmacological effects (Motterlini et al., 2000; Ringman et al., 2005; Heneka and O'Banion, 2007). Curcumin has also been used in the prevention and treatment of Alzheimer's disease (AD) (Xiong et al., 2011; Belkacemi et al., 2011; Huang et al., 2012), but the mechanism remains elusive. Epidemiological studies have shown that India has the lowest incidence of AD in the world (Wang and Du, 2003; Perry and Howes, 2011). One potential reason for this may be the fact that curcumin is the main component of curry that is widely consumed in India. An *in vitro* study revealed that curcumin has anti-amyloid β (Aβ) aggregation activity, antioxidation properties, inhibits β-secretase and acetylcholinesterase, and inhibits Aβ-induced inflammation in Aβ-induced AD rats (Giri et al., 2004). *In vivo* studies also demonstrated that curcumin can prevent Aβ aggregation as well as phosphorylation of the tau protein in the brain (Lim et al., 2001). These results indicate that curcumin may be one of the most promising compounds for treating AD.

Inhibitory factors of axonal regeneration play an important role in the occurrence and development of AD (Hardy and Selkoe, 2002). The Nogo receptor (NgR) is the co-receptor of three axonal growth inhibitory proteins (Nogo-A, MAG, and OMgp) (McGee and Strittmatter, 2003). NgR can inhibit axonal growth by activating downstream intracellular signaling molecules, the small GTP enzyme RhoA and its effector, Rho-kinase (ROCK) (Fournier et al., 2003). A previous study (Gil et al., 2006) showed that Nogo-A and NgR were involved in the pathological process of AD. Nogo-A is overexpressed in the hippocampus of AD patients, and can be deposited in senile plaques along with Aβ. Xiao et al. (2012) demonstrated that NgR could not only inhibit axonal regeneration by activating ROCK, but could also promote Aβ accumulation. Although several studies have demonstrated the potential of curcumin in treating AD, its mechanism is seldom reported, particularly with respect to its effect on axonal regeneration. In this study, effects of curcumin on spatial learning and memory ability and on NgR expression and axonal growth were investigated in Aβ1-40-induced AD rats. A correlation analysis between NgR expression and axonal growth was conducted. The objective of this study was to provide a theoretical basis for the treatment of AD with curcumin.

MATERIAL AND METHODS

Reagents and apparatus

The main experimental reagents and apparatus were as follows: Aβ1-40 peptides

(Sigma, USA), curcumin (Sigma), goat anti-NgR antibody and mouse anti-Neurofilament 200 kDa antibody (dilution of 1:100 and 1:200, respectively; Wuhan Boster Biological Technology, China), Morris water maze and brain stereotaxic instrument (Narishige, Japan), gel imaging system (Bio-Rad, USA), BS-50 optical microscopy (Olympus, German), CM-2000B image analysis system (Beijing University of Aeronautics and Astronautics, China), reverse transcription-polymerase chain reaction (RT-PCR) kits (Takara Biotechnology, Co., Ltd., Dalian, China), NgR primers (upstream sequence: 5'-TGC TGG CAT GGG TGT TAT GG-3', downstream sequence: 5'-CGG AAG GTG TTG TCG GGA AG-3', length of amplified fragment: 493 bp), β-actin (upstream sequence: 5'-CGT AAA GAC CTC TAT GCC AAC A-3', downstream sequence: 5'-CGG ACT CAT CGT ACT CCT GCT-3', length of amplified fragment: 229 bp).

Animals and groupings

Fifty-three healthy male rats (230-250 g) were provided by the Experimental Animal Center of Chongqing Medical University, China. After 1 week of adaptive feeding, the Morris water maze was used to select rats with no abnormalities. Any rat that could not find the hidden platform after 2 min in two of four quadrants was regarded as a cognitive disorder rat (N = 5). The remaining 48 rats were randomly divided into the sham-operated group, the AD group, and the curcumin group, with 16 rats in each group. The curcumin group was treated with intraperitoneal injections of 300 mg/kg curcumin dissolved in dimethyl sulfoxide (DMSO). The sham-operated and AD groups were treated with equal amounts of DMSO. Injections were conducted once a day for 7 consecutive days (Thiyagarajan and Sharma, 2004).

Constructing the animal model

Aβ1-40 solution (1 mg/μL) was prepared with sterile normal saline, and then incubated at 37°C for 1 week to build neurotoxic oligomers. After abdominal anesthesia with 3.5% chloral hydrate, rats were fixed in the stereotaxic instrument, and the bregma was exposed via a skin incision. A craniotomy was performed using a dental drill in area CA1 of the hippocampus (3.0 mm posterior to the bregma and 2.2 mm lateral to the midline), according to the publication "Rat Brain in Stereotaxic Coordinates" (Paxinos and Watson, 1998). Subsequently, 10 μL Aβ1-40 solution was slowly injected (2.8 mm under the cortex). The syringe needle was retained for 10 min in order to fully disperse the solution. The sham-operated group was treated with injections of equal amounts of normal saline. After injection, rats in each group were fed in a single cage until completely awake.

Morris water maze testing

The learning and memory abilities of rats were tested in a Morris water maze after 1 month of modeling (Nakamura et al., 2001).

1) Place navigation. Rats were trained to find the hidden platform. The learning and memory abilities of rats were tested by measuring the escape latency (time of finding the hidden platform within 2 min). The rat was placed into the swimming pool with its head facing the pool wall from the two entries, respectively. The escape latency and the swimming path

were then recorded. If the rat did not find the platform within 2 min, it was towed to the hidden platform by a stick for a 10 s stay, and the escape latency was recorded as 120 s. The experiment was performed twice a day for 5 consecutive days.

2) Spatial probe. The hidden platform was removed on the sixth day. The rat was put into the swimming pool from either of the two entries. The escape latency and swimming path were recorded. The percentage of residence time in each quadrant was calculated.

Specimen preparation

After Morris water maze testing, 8 rats in each group were treated with abdominal anesthesia using 3.5% chloral hydrate. The right hippocampus was removed on an ice pad after rapid decapitation and divided into two parts. The hippocampus tissue was placed in a freezing tube, which was disinfected with diethylpyrocarbonate overnight. Tissues were then frozen in liquid nitrogen for 2-3 h, and then stored at -80°C until testing. The remaining rats in each group were treated with intracardiac perfusion with 4% paraformaldehyde buffer solution for fixing. The hippocampus was taken out, and 4 μm-thick paraffin sections were prepared, which were hematoxylin and eosin-stained for immunohistochemical examinations.

Determination of the expression of NgR mRNA

The RT-PCR method was used to determine the expression of NgR mRNA. Total RNA was extracted from the hippocampal tissue using Trizol (Chomczynski, 1993). The reverse transcription reaction parameters were as follows: 30°C for 10 min, 50°C for 30 min, 99°C for 5 min, and 5°C for 5 min, followed by instantaneous centrifugation. The PCR was run in a total volume of 40 μL, including 10 μL cDNA, obtained from the reverse transcription reaction, as template, 0.5 μL upstream primer, and 0.5 μL downstream primer. The amplification parameters were as follows: 94°C for 3 min, then 35 cycles (94°C for 30 s, 54°C for 30 s, 72°C for 36 s), followed by 72°C for 5 min. Semi-quantitative analysis was conducted on the products using the Quantity One-4.4.0 software.

Determination of the expression of the NgR and axonal proteins

The immunohistochemical method was used to determine the expressions of the NgR and axonal proteins. The hippocampus paraffin sections were de-waxed and rehydrated in water. After microwave antigen repair, they were incubated in goat anti-NgR and mouse anti-Neurofilament 200 kDa antibody at 4°C overnight. For the negative control, 0.01 M phosphate-buffered saline was used instead of primary antibody. The sections were stained with 3,3'-diaminobenzidine (DAB). After dehydration and sealing, the sections were observed under an optical microscope. Brown granules indicated positive immunoreactive cells.

Statistical analysis

Data are reported as means ± SE. Statistical analysis was performed using the SPSS 11.0 statistical software. Comparisons between two groups were performed using a single factor analysis of variance. $P < 0.05$ was considered as statistically significant.

RESULTS

Morris water maze testing

As shown in Table 1, the escape latency in each group shortened gradually after the experiment started. This indicated that the rat learning ability to find the platform was improved by previous training. From the second day, the escape latency in the AD group was significantly longer than that in the sham-operated group ($P < 0.05$), and the majority of rat behavior was edging and random. This indicated that the AD model was successfully constructed. After the platform was removed, the residence time in quadrant II in the AD group was significantly longer than that in the sham-operated control group and the curcumin group, respectively ($P < 0.05$) (Table 2). This indicated that the learning and memory abilities of rats in the AD group were clearly reduced. There was no significant difference in learning and memory abilities of rats between the curcumin group and the AD group ($P > 0.05$). Therefore, curcumin could significantly improve the learning and memory abilities of cognitive disorder rats.

Table 1. Escape latency in each group.

Group	Escape latency (s, means ± SE)				
	1 day	2 days	3 days	4 days	5 days
Sham-operated group	66.3 ± 13.2	40.1 ± 11.6	24.3 ± 8.63	16.7 ± 8.2	12.6 ± 5.2
AD group	98.7 ± 11.3[a]	87.2 ± 12.4[a]	57.6 ± 12.5[a]	46.3 ± 12.7[a]	34.7 ± 11.4[a]
Curcumin group	67.2 ± 14.5[b]	42.2 ± 11.9[b]	25.6 ± 9.1[b]	18.2 ± 7.9[b]	13.6 ± 5.8[b]

[a]$P < 0.05$ (between AD group and sham-operated group). [b]$P > 0.05$ (between curcumin group and sham-operated group).

Table 2. Percentage of the residence time in each group.

Group	Quadrant (%, means ± SE)			
	I	II (original quadrant)	III	IV
Sham-operated group	20.72 ± 6.53	42.45 ± 7.81[a]	19.57 ± 3.40	17.53 ± 7.80
AD group	27.67 ± 9.43	28.83 ± 5.18	23.73 ± 4.29	23.15 ± 5.09
Curcumin group	21.75 ± 5.34	41.32 ± 8.12[b]	17.43 ± 4.83	19.66 ± 8.94

[a]$P < 0.05$ (between AD group and sham-operated group). [b]$P > 0.05$ (between curcumin group and sham-operated group).

Effects of curcumin on hippocampal expression of NgR mRNA and protein

The positive reactive part of NgR, which appeared brown under the light microscope, was located on the neuron membrane. A small amount of NgR protein expression was observed in the sham-operated group. The expression levels of NgR mRNA and protein in the AD group were significantly higher than those in the sham-operated group, respectively ($P < 0.05$). The number of positive cells in the AD group increased and its color was enhanced compared to the sham-operated group. After treatment with curcumin, the expressions of NgR mRNA and protein were significantly reduced ($P < 0.01$ and $P < 0.05$, respectively) (Table 3 and Figure 1A-C).

Table 3. Hippocampal expressions of NgR mRNA, NgR protein and axon protein in rats (means ± SE).

Group	Sham-operated group	AD group	Curcumin group
Expression of NgR mRNA	1.1146 ± 0.0114[a]	1.5628 ± 0.0326	1.3058 ± 0.0671[b]
Expression of NgR protein	0.2366 ± 0.0243[a]	0.5642 ± 0.0143	0.3513 ± 0.1173[a]
Expression of axon protein	0.2138 ± 0.0281[a]	0.1142 ± 0.0164	0.1738 ± 0.0176[b]

[a]$P < 0.05$ (compared to the AD group). [b]$P < 0.01$ (compared to the AD group).

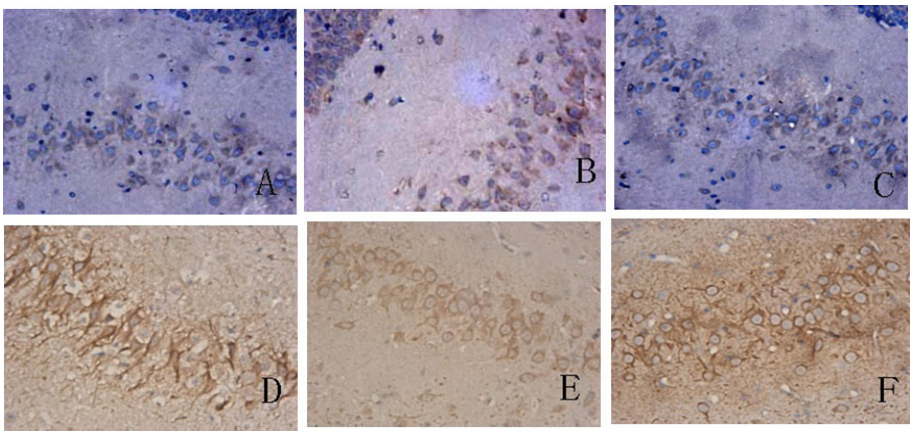

Figure 1. Expression of the NgR protein in rats (DAB, 400X). **A.** Sham-operated group; **B.** Alzheimer's disease group; **C.** Curcumin group; **D.** Sham-operated group; **E.** Alzheimer's disease group; **F.** Curcumin group.

Effects of curcumin on hippocampal expression of axon protein

Immunohistochemical results showed that the morphology of hippocampal neurons in the sham-operated group was normal, showing axon expression in almost all neurons. The expression of axon protein in the AD group was significantly less than that in the sham-operated group ($P < 0.05$). After treatment with curcumin, the expression of axon protein significantly increased ($P < 0.05$) (Table 3 and Figure 1D-F).

Correlation between NgR expression and axonal growth

There was a negative correlation between the hippocampal expression of NgR mRNA and axonal growth ($r_1 = -0.66707$, $P < 0.01$), and between NgR mRNA protein expression and axonal growth ($r_2 = -0.41136$, $P < 0.05$) in each group (Figures 2 and 3).

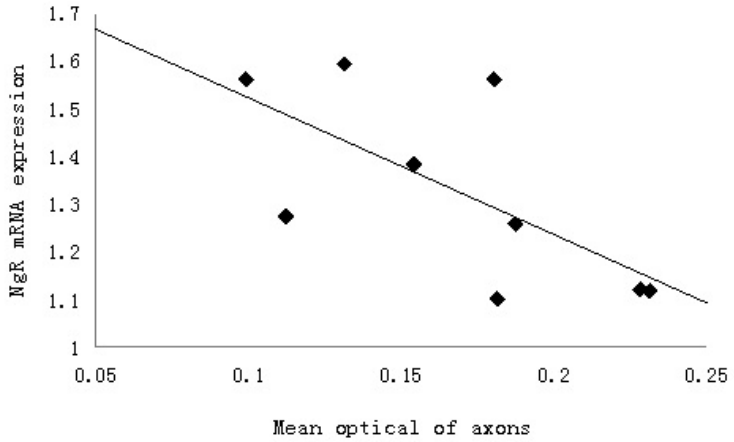

Figure 2. Correlation between the expression of NgR mRNA and axonal growth in each group.

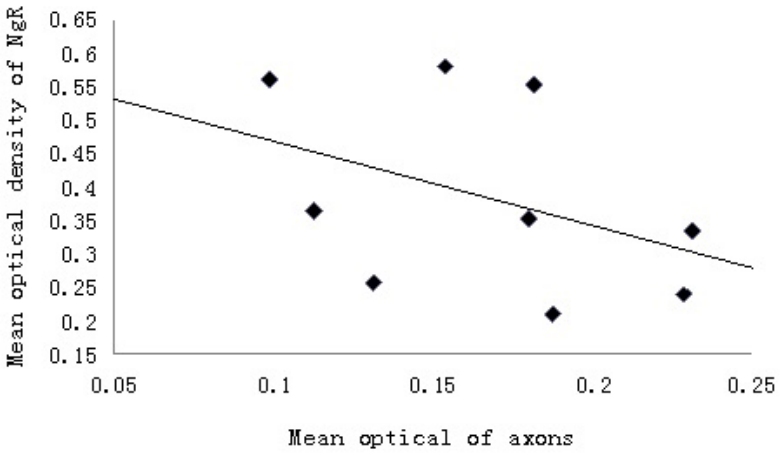

Figure 3. Correlation between the expression of NgR protein and axonal growth in each group.

DISCUSSION

The hippocampus plays a key role in learning and memory. In this study, rats treated with hippocampal injection of Aβ1-40 showed obvious signs of cognitive disorder, which indicated that the AD model was successfully constructed. Although the spatial learning and memory abilities in the curcumin group were lower than those of the sham-operated group, they were significantly higher than those of the AD group. Therefore, curcumin can clearly improve the spatial learning and memory abilities of Aβ-induced cognitive disorder rats. These results are consistent with the study of Lim et al. (2001).

Zhu et al. (2007) found that, in AD patients, NgR expression of pyramidal neuron cell bodies and neurites in the hippocampus significantly increased compared with expression levels in the normal hippocampus. The proportion of positive NgR neurons in all detected pyramidal cells also significantly increased in area CA1-CA2, but not in area CA3-CA4. In addition, immunohistochemical results showed that, in area CA1, the content of hyperphosphorylated tau protein (AT-8 positive) in the neurons' cytoplasm significantly increased and was co-expressed with NgR; however, this co-expression was not observed in neuritic plaques. These results suggested that NgR might be involved in the formation of neuronal fiber entanglement during the pathogenesis of AD, which was further confirmed in the present study. In the AD group, the expressions of NgR mRNA and protein significantly increased. The NFP-200-positive fiber of axonal protein was disorganized and irregular, and its numbers were remarkably reduced. After treatment with curcumin, the expressions of NgR mRNA and protein significantly decreased, and the expression of axonal protein significantly increased, showing larger numbers, less bifurcation, and a shape tending to normal. Furthermore, there was a negative correlation between NgR expression and axonal growth, which suggested that curcumin could inhibit the hippocampal expression of NgR while promoting axonal growth in AD rats.

Excessive expression of NgR has been shown to prevent α- and β-secretase from cleaving amyloid precursor protein (APP), which reduces the production of Aβ (Park, 2006b).

Aβ/APP metabolism is a complex multi-step process involving a variety of proteins. Therefore, results of the peripheral administration of soluble NgR fragments in AD rats (Burdick et al., 1992; Park, 2006a) are not contradictory with results of our study. This suggests that both excessive and insufficient expression of NgR is not favorable for axonal regeneration. The precise level of NgR expression that should be maintained to improve cognitive function requires further investigation. Nonetheless, results of the present study demonstrated that curcumin might improve the cognitive function of AD rats by lowering the hippocampal expression of NgR and promoting axonal regeneration.

The AD pathology mechanism and treatment method are currently under investigation. Based on its chemical characteristics and results of clinical applications, it appears that curcumin is an effective therapeutic drug with promising prospects for treating AD (Cole et al., 2004; Wang et al., 2012; Jiang et al., 2012). The mechanism of action of curcumin and its target are very complex, and need further investigation. The present study is the first to show that curcumin can improve cognitive function of AD rats by inhibiting the expression of NgR. This provides a new perspective for clinical treatments of Aβ-induced cognitive disorders. Because the majority of data concerning the efficacy of curcumin are derived from cell and animal experiments, more clinical data are required to confirm our results, which is a focus of our future research.

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