Study on the molecular mechanisms of the inflammation induced by β-amyloid in vivo

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Abstract: *Background*. The study is to explore the molecular mechanisms of the inflammation induced by β-amyloid (Aβ) in the tissue of cortex and hippocampus of mice. *Methods*. The tissue of cortex and hippocampus of mice, were exposed to $A\beta_{1-42}$ with or without Pyrrolidinedithiocarbamate ammonium (PDTC), then Enzyme linked immunosorbent assay (ELISA) for interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα); Quantitative real time polymerase chain reaction (RT-PCR) for IL-1β and TNFα mRNA, western blot analyses of IκBα and NF-κB p65. *Results*. PDTC inhibited the protein expression of IL-1β, TNFα, IL-1β and TNFα mRNA, inhibited the decreasion of IκBα in the cytoplasm, and also inhibited elevation of NF-κB p65 in the nucleus in Aβ-stimulated tissue of cortex and hippocampus of mice. *Conclusion***.** Our findings suggest that the IL-1β, TNFα protein and mRNA were induced by Aβ via the NF-κB signal pathway in the tissue of cortex and hippocampus of mice.
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1. Introduction

AD is a progressive neurodegenerative disease characterized by cognitive impairment. Even after centuries of research and exploration, the exact etiology of AD remains to be determined, so, there is no effective treatment until now. The risk of AD increases with age, and it reduced the quality of life of people. With the rapid expansion of aged population, AD will be one of the most common cause of death. Therefore, it is imperative to study the pathogenesis in depth. In recent years, studies increasingly indicate that inflammation contributes to the progression of AD (Hoozemans JJ, 2006; Eikelenboom P, 2006), and in the pathogenesis of AD, Aβ plays an important role. Aβ activates microglia, and the activated microglia promotes neuronal injury through the release of proinflammatory factors (Graeber MB, 2010; Moore AH, 2002; Wang MJ, 2002). In this study, the proinflammatory effect of Aβ on the cortex and hippocampus of mice of and the possible signal transduction pathways were discussed.

2. Subjects and Methods

2.1 Reagents

NF-κB p65 antibodies were from Cell Signaling Technology Inc.(Beverly, MA, USA), IκBα antibodies were from Abcam Inc.(Cambridge, MA, UK), PDTC was from Sigma-Aldrich Co. LLC.(Ronkonkoma, America), goat anti-rabbit IgG-PE was from Santa Cruz Biotechnology (Santa Cruz, CA), goat anti rabbit IgG (H+L) HRP was from Thermo Fisher Scientific Inc. (Rockford, IL, USA), IL-1β, TNFα ELISA assay kits were purchased from Wuhan Boster Bioligical Technology, LTD (Wuhan, Hubei, China).

2.2 Preparation of $A\beta_{1-42}$

$A\beta_{1-42}$, obtained from AnaSpec, Inc (Fremont, CA, USA), was dissolved in sterile double-distilled water to a concentration of 0.1 mM, incubated for 5 days at 37°C to allow fibril formation, and then further diluted in PBS to 5 μM.

2.3 Animals and Intracerebroventricular injection (ICV)

The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji Medical College, Huazhong University of Science and Technology, which abides by the Institute of Laboratory Animal Resources (ILAR) guide. The intracerebroventricular (ICV) infusion method was established by Laursen and Belknap (Laursen SE, 1986). Five groups of 3-4 month male C57BL/6 mice weighing 20~25g were anesthetized and administrated with ICV infusion of $A\beta_{1-42}$ 50 μg with and without the presence of PDTC (pretreated with PDTC at 5μg, 50μg for 30 min) into mice. Each group of the animals consisted of 5 mice. The control group were injected with physiological saline, and administrated with ICV infusion for seven consecutive days. The mice were sacrificed at the 8th day after the ICV infusion. Sections of cortex and hippocampus of the mice were collected.

2.4 Enzyme linked immunosorbent assay (ELISA) for IL-1β and TNFα

IL-1β and TNFα, which existed in the tissue homogenate, were measured by ELISA kit according to the manufacturer's instructions. The optical density was measured at 450 nm using a microplate reader, and the values were obtained from standard curves using recombinant IL-1β and TNFα in the ELISA kits.

2.5 Quantitative real time polymerase chain reaction (RT-PCR) for IL-1β and TNFα mRNA

Total RNA was extracted from tissues using TRIzol Reagen (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the instructions. Each reverse transcription reaction was made to a final volume of 25 μl with the following reagents: 1 μg of total RNA and 1μl of M-MLV reverse transcriptase (Invitrogen), 4 μl of M-MLV RT 5 × buffer, 20 U of RNasin, 1 μl of Random primer (10 pmol/μl),1 μl of dNTP mix (10 mM) and DEPC water. They were mixed and incubated at 37°C for 50 min and at 70°C for 15 min. The cDNA thus obtained was then stored at -20°C. The real time RT–PCR was carried out using LightCycler DNA master plus SYBR Green I kit (TOYOBO Co., LTD, Osaka, Japan) according to the protocols. After an initial denaturation step (95°C for 1 min), the PCR was performed as follows: 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 45 s. Primer sequences (Invitrogen) were as follows (orientation 5′ to 3′): β-actin (forward): CCGTGAAAAGATGACCCAG, β-actin (reverse): TAGCCACGCTCGGTCAGG; IL-1β (forward): TTTGAAGTTGACGGACCCC, IL-1β (reverse): GTGCTGCTGCGAGATTTGA; TNFα (forward): CGGGCAGGTCTACTTTGGAG, TNFα (reverse): CAGGTCACTGTCCCAGCATC. For the relative comparison of each gene, the data of real time PCR were analyzed with ΔΔCt method.

2.6 Western blot analyses of IκBα and NF-κB p65

After the tissues were resuspended in the lysis buffer, and the nuclear protein and the cytoplasmic protein were extracted. The protein samples were electrophoresed by SDS-PAGE gel, and transferred to a nitrocellulose membrane, and assayed firstly using rabbit polyclonal antibodies against IκBα or NF-κB p65, then with anti-rabbit horseradish peroxidase-coupled secondary antibody, and at last with an enhanced chemiluminescence detection reagent.

2.3 Statistical analysis

Data were subjected to statistical analysis using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). Data are shown as means±S.D. for at least three independent experiments. Statistical significance was analyzed by one-way ANOVA, followed by the Scheffe's test for comparison of multiple comparison. Difference with p value < 0.05 was considered statistically significant.

3. Results

3.1 PDTC inhibited IL-1β and TNFα production stimulated by Aβ in the tissue of cortex and hippocampus of mice

In this study, we investigated whether PDTC inhibits the production of IL-1β and TNFα stimulated by Aβ in the cortex and hippocampus of mice. The effects of PDTC on the production of IL-1β and TNFα are depicted in (Fig 1). The expression of IL-1β and TNFα markedly increased in the cortex and hippocampus of mice exposed to Aβ. In contrast, when pretreated with PDTC before being exposed to Aβ, their production was found to greatly decrease. PDTC alone did not affect the production of IL-1β and TNFα. Analysis showed that IL-1β and TNFα production stimulated by Aβ in the cortex and hippocampus of mice could be inhibited by PDTC.

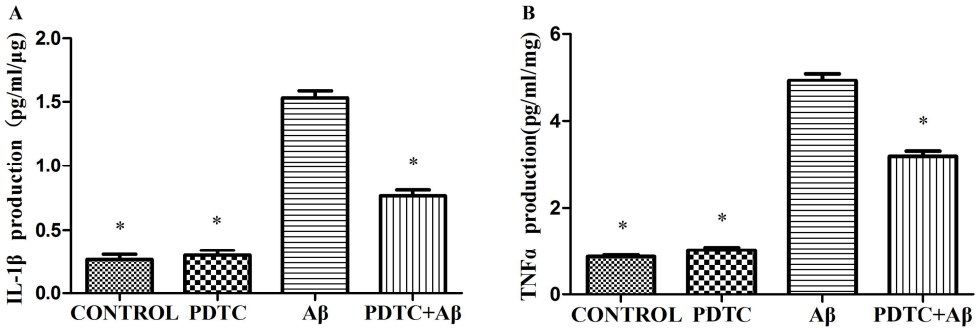

Fig 1 ELISA assay for IL-1β and TNFα protein after the tissue of cortex and hippocampus of mice were treated with or without Aβ in the absence or presence of PDTC. Aβ markedly enhanced the protein expression of IL-1β and TNFα, PDTC alone did not affect protein expression. PDTC significantly inhibited the protein expressions of IL-1β and TNFα induced by Aβ (Fig 1 A, B), Data are presented as means±S.D. *P < 0.05, **P < 0.01 compared with Aβ group.

3.2 PDTC attenuated the gene expressions of IL-1β and TNFα stimulated by Aβ in BV2 cells and tissue in the cortex and hippocampus of mice

We next analyzed the influence of PDTC on the gene expression of IL-1β and TNFα (Fig 2). Aβ markedly up-regulated the IL-1β and TNFα mRNA level, while PDTC significantly down-regulated the mRNA level of IL-1β and TNFα induced by Aβ. PDTC alone did not affect the IL-1β and TNFα mRNA level. These observations are consistent with the expression of IL-1β and TNFα proteins, and the results imply that PDTC inhibits IL-1β and TNFα protein expression by directly modulating gene transcription.

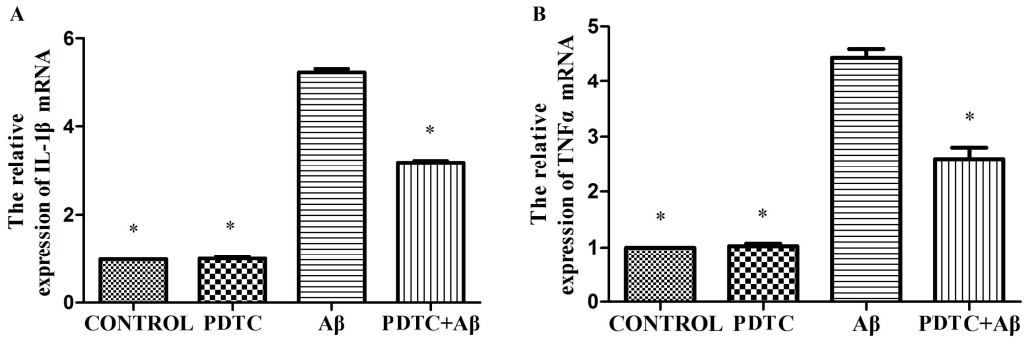

Fig 2 RT-PCR assay for IL-1β and TNFα mRNA after the tissue of cortex and hippocampus of mice were treated with or without Aβ in the absence or presence of PDTC. Aβ markedly enhanced the gene expression of IL-1β and TNFα, PDTC alone did not affect gene expression. PDTC significantly attenuated the gene expressions of IL-1β and TNFα induced by Aβ (Fig 2 A, B), Data are presented as means±S.D. *P < 0.05, compared with Aβ group.

3.3 PDTC blocked IκBα degradation and NF-κB p65 subunit translocation into the nucleus stimulated by Aβ in tissue in the cortex and hippocampus of mice

This part of the study investigated whether PDTC acts to block the activation of the NF-κB pathway. The effects were determined by western blot (Fig 3) The results indicate that the degradation of IκBα , which is a key step in the regulation of the NF-κB pathway, can be inhibited by PDTC, and the following translocation of NF-κB p65 subunit was decreased, which means that only a few DNA binding forms of NF-κB could translocate into the nucleus where it binds to its recognition sites in the upstream region of genes of IL-1β and TNFα. Thus, the gene expressions of IL-1β and TNFα were attenuated.

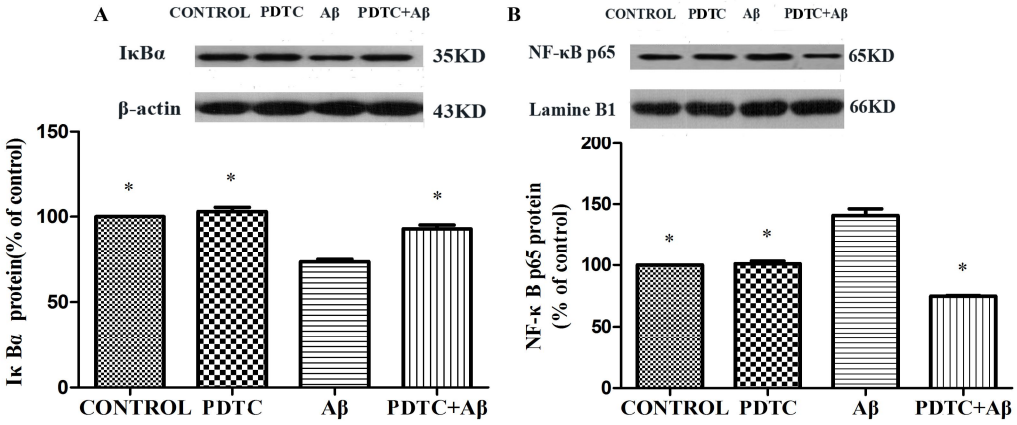

Fig 3 Western blot analyses of IκBα and NF-κB p65 after the tissue of cortex and hippocampus of mice were treated with or without Aβ in the absence or presence of PDTC. Plasmic extracts or nuclear extracts were separated and blotted sequentially with the indicated antibodies to evaluate both IκBα protein contents in cytoplasm and NF-κB p65 protein contents in nucleus. Aβ caused a marked degradation of IκBα, and then induced the NF-κB p65 subunit to enter the nucleus, these reactions could be inhibited by PDTC (Fig 3 A, B).The data represent the mean±SD of at least three independent experiments. *P < 0.01 compared with Aβ group.

4. Discussion

AD, a progressive neurodegenerative disease, is the most common causes of dementia, affecting a large number old people in the world. With the aging population increasing promptly, patients, families and society are facing more and more problems associated with AD in China. The presence of senile plaques and neurofibrillary tangles is the pathological hallmarks in AD. The plaques contain extracellular deposits of β-amyloid (Aβ) surrounded by damaged neuronal processes and reactive glia, and the hyperphosphorylated tau proteins accumulates intracellular as neurofibrillary tangles. Multiple studies suggest that pathological changes of AD is the result of cumulative damage of inflammation (Wyss-Coray T, 2006). Some clinical study indicate that the levels of IL-1β、IL-6 and TNFα elevate in the AD brain (Candore G, 2007; Candore G, 2004; Tarkowski E, 1999; Frei K, 1989; Griffin WS, 1995)，and the anti-inflammatory drug down-regulates the expression of IL-1β and TNFα (Jun Wu , 2011). Some study report that nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the risk for AD, the relative risk of AD was 0.95 in subjects with 1 month use of NSAIDs, 0.83 in those with 1 month - 1 year use, and 0.20 in those with 2 years use (Bas A. in t' Veld BA, 2001).；Other studies indicate that the incidence of AD decreased after taking NSAIDs (Breitner JC, 2003; Etminan M, 2003; McGeer PL, 1996). These provide very strong evidence that that inflammation contributes to the progression of AD. Being part of the complex biological response of tissues to pathogens, inflammation exists in the pathological process of variety of diseases. They are an important and necessary part of the normal host responses to harmful stimuli, but persistent inflammatory response and the overproduction of inflammatory molecules causes severe damage to organs and tissues (Licastro F, 2005), and may aggravate immune-inflammatory-related disease. Compelling evidence from basic and clinical research studies has supported the conclusion that neuroinflammation induced by Aβ is associated with the development of AD neuropathology (Wyss-Coray T, 2006)，Aβ self-aggregate to form insoluble fibrils, deposit as plaques (Pereira C, 2005), activate microglia, the resident macrophages of the brain, and these activated microglia (Rogers J, 2001) may then promote neuronal injury through the release of proinflammatory and cytotoxic factors, including IL-1, IL-6 and TNFα (Candore G, 2007; Hanisch UK, 2002; Minghetti L, 1998; McGeer EG, 1998; Mrak RE, 1995; Griffin WS, 1998; Akiyama H, 2000; Town T, 2005; Combs CK, 2001; Meda L, 2001). IL-1 is an early response cytokine which is overexpressed in brains of AD and associated with neuritic plaque (Griffin WS, 1998; Griffin WS, 2002; Sheng JG, 1996). A large number of IL-1 increased the production and enhance the activity of neuronal acetylcholinesterase, leading to dysfunction of the cholinergic system and memory impairment of AD (Li Y, 2000). IL-6 promote enhanced Aβ1-40 and Aβ1-42 production，reverse the neuroprotective effect of soluble amyloid precursor protein (sAPP) (Ringheim GE, 1998; Del Bo R, 1995). High levels of IL-6 induce hippocampal neuronal degeneration and damage cholinergic neural pathways seriously, resulting in impairments in learning and memory in the central nervous system of transgenic mice (Campbell IL, 1997). TNF α up-regulate the expression of COX-2 , leading to free radicals generation and subsequent nerve cells damage (Culpan D, 2003). TNFα enhanced the sensitivity of neurons to free radicals, leading to neuronal degeneration (Combs CK, 2001). TNFα also stimulate the synthesis of Aβ (Blasko I, 1999). In summary, inflammatory molecules play an important role in the process of amyloid plaques and the neurofibrillary tangles formation，neuronal damage，memory and cognitive impairment.

Our study found that，the degradation of IκBα and NF-κB p65 subunit translocation into the nucleus stimulated by Aβ, and the following expression of inflammatory factors in tissue in the cortex and hippocampus of mice can be inhibited by PDTC, the specific inhibitors of NF-κB signaling. These findings suggest that the IL-1β, TNFα protein and mRNA were induced by Aβ via the NF-κB signal pathway in the tissue of cortex and hippocampus of mice.

NF-κB is a nuclear factor that bound selectively to the κ enhancer and was found in extracts of B-cell tumors, it bound only to the κ light-chain enhancer, and it covered the sequence GGGACTTTCC (Sen R, 1986). NF-κB plays a key role in inflammatory processes. IkBα is the inhibitor of NF-κB (Baldwin AS, 1996; Ghosh S, 1998), and it bind to NF-κB (p65/p50 dimer) retained within the cytoplasm, preventing NF-κB importing into the nucleus. IkBα (Ghosh S, 1998; Karin M, 2000; Silverman N, 2001; Ghosh S, 2002; Huxford T, 1998; Jacobs MD, 1998). Multiple studies have found that a variety of inflammatory cytokines, including TNF and IL-1 family members can activate NF-κB signal pathway (Ghosh S, 1998; Siebenlist U, 1994; Pahl HL,1999), thereby, Aβ can active NF-κB signaling pathway, and induce further activation, resulting in a detrimental cycle, thus, that would exacerbate nerve cells and tissue damage. Some studies show that, the senile plaques and neurofibrillary tangles, as the pathological hallmarks of AD, are all related to activation of NF-κB (Yamamoto Y, 2001; Kaltschmidt B, 1999). This imply that inhibition of NF-κB activation, not only significantly suppressed the Aβ-induced inflammatory response, but also reduced

the intracellular neurofibrillary tangles and extracellular deposition of Aβ, and reduced the risk of cognitive decline. As the main therapeutic target, inhibition of NF-κB activation has become a key therapeutic target for treatment of AD, and is likely to have far-reaching consequences.

5. Conclusions

IL-1β, TNFα protein and mRNA were induced by Aβ via the NF-κB signal pathway in the tissue of cortex and hippocampus of mice and BV2 microglial cells.

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