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Anti-neuroinflammation activity of acetylpuerarin mediated by a PKC-δ-dependent caspase signaling pathway: *in vivo* and *in vitro* studies

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Objective: This study was performed to evaluate the regulating effects of acetylpuerarin on inflammation in an Alzheimer's disease (AD) rat model and an inflammatory cell model. *Methods:* Healthy female Wistar rats and mouse BV2 microglia cells were selected. AD rat models were established with the method of bilateral intrahippocampal amyloid-β (Aβ)1–42 injections and the inflammatory cell models were established using $A\beta_{25\text{-}35}$-induced mouse BV2 microglia cells. The cytotoxicity of acetylpuerarin on BV2 microglial cells was detected by MTT assay and the morphological changes of BV2 microglia cells were observed under inverted phase contrast microscope. As inflammatory parameters, the expressions of IL-1β, iNOS, IL-6 and TNF-α were examined by Elisa, Immunohistochemistry, Quantitative real-time PCR (qRT-PCR), Western blot and Immunofluorescence analyses. We also examined the acetylpuerarin's effect on the activity of PKC-δ, IKKβ and caspase-8/caspase-3 pathway. *Results:* Acetylpuerarin exerted no significant cytotoxicity on BV2 microglia cells and was applied in all subsequent experiments. Acetylpuerarin treatment mitigated $A\beta_{25\text{-}35}$-induced morphological changes associated with microglia activation. Moreover, the expressions of caspase-8, cleaved caspase-3, PKC-δ, IKKβ, iNOS, IL-1β and TNF-α in $A\beta_{25\text{-}35}$-stimulated BV2 microglia cells were significantly suppressed by acetylpuerarin and in a dose-dependent manner. Additionally, the expression of IL-1β in hippocampus and the level of IL-6 in serum of $A\beta_{1\text{-}42}$ treated rat were reduced by acetylpuerarin and in a concentration-dependent manner. *Conclusion:* Our results suggest that acetylpuerarin's anti-inflammation mechanism on AD may be mediated through the PKC-δ-dependent caspase signalling pathway.

1. Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder characterized by extracellular β-amyloid (Aβ) depositions in the form of neurotic plaques and appearance of intraneuronal hyperphosphorylated tau protein in the form of neurofibrillary tangles (NFTs), is now becoming a growing problem which affects most of the elderly people around the world. Regarding AD's pathophysiology, the amyloid cascade hypothesis has been the major pathogenic concept in the field of AD research for the past few decades. At present, pro-longed microglial activation and subsequent production of neurotoxic pro-inflammatory molecules (interleukin-1β, IL-1β; tumor necrosis factor-α, TNF-α; interleukin-6, IL-6 and so on) are thought to contribute to AD's occurrence and exacerbate AD's progression (Heneka et al. 2015; Heppner et al. 2015). Control of the inflammatory reaction would therefore be an effective therapeutic option for treatment of AD.

Acetylpuerarin (AP) is a newly modified isoflavonoid compound derived from the Chinese medical herb puerarin, which has higher liposolubility than puerarin and can cross the blood-brain barrier (BBB) easily (Xiang et al. 2014) (Fig. 1a-b). Previous studies have shown that acetylpuerarin could protect against brain injury in rats by scavenging oxygen free radicals, increasing the activity of antioxidase, promoting cell survival in inflammatory areas in vivo, inhibiting hippocampal neurons degeneration, decreasing calcium concentration in neurons, reducing apoptosis in hippocampal neurons (Hou et al. 2007; Liu et al. 2013, 2007). In addition to these beneficial actions, acetylpuerarin has been noted for its anti-inflammatory properties. It can attenuate the activation of immune cells and the subsequent synthesis and release of pro-inflammatory mediators such as nitric oxide (NO) through the inhibition of inducible nitric oxide synthase (iNOS) expression (Li et al. 2005), inhibit arachidonic acid (AA)-metabolizing enzymes and AA metabolites via downregulation the expression of group V sPLA2 and phosphorylation of ERK1/2, cPLA2α, and expression of transcription factor, NF-κB in LPS-induced primary rat astrocytes (Xiang et al. 2014). Our previous study has found that acetylpuerarin can improve Aβ-induced AD rats' learning and memory deficits and suppress the levels of protein kinase C delta (PKC-δ); IkB kinaseβ (IKKβ), and iNOS in the hippocampus, which indicates it's neuroprotective functions and anti-neuroinflammatory effects (Meng et al. 2013). However, the precise mechanisms for its anti-inflammatory activity are still not fully understood. Hence, the present study was designed to observe whether acetylpuerarin's anti-inflammation effects is mediated through the PKC-δ-dependent cysteinyl aspartate specific proteinase (caspase) signalling pathway in $A\beta_{25\text{-}35}$-stimulated BV2 microglia cells and $A\beta_{1\text{-}42}$-treated rats.

2. Investigations and results

2.1. Effect of acetylpuerarin on BV2 microglia cells viability

Evaluation of the cytotoxicity of acetylpuerarin was imperative before further studies were carried out. The effect of acetylpuerarin on BV2 cells viability was determined with or without $A\beta_{25\text{-}35}$ by MTT assay. The results revealed that acetylpuerarin did not decrease the viability of BV2 cells when they were incubated in the presence or absence of $A\beta_{25\text{-}35}$ (20 μM). Namely, acetylpuerarin showed no cytotoxicity on BV2 cells (Fig. 2).

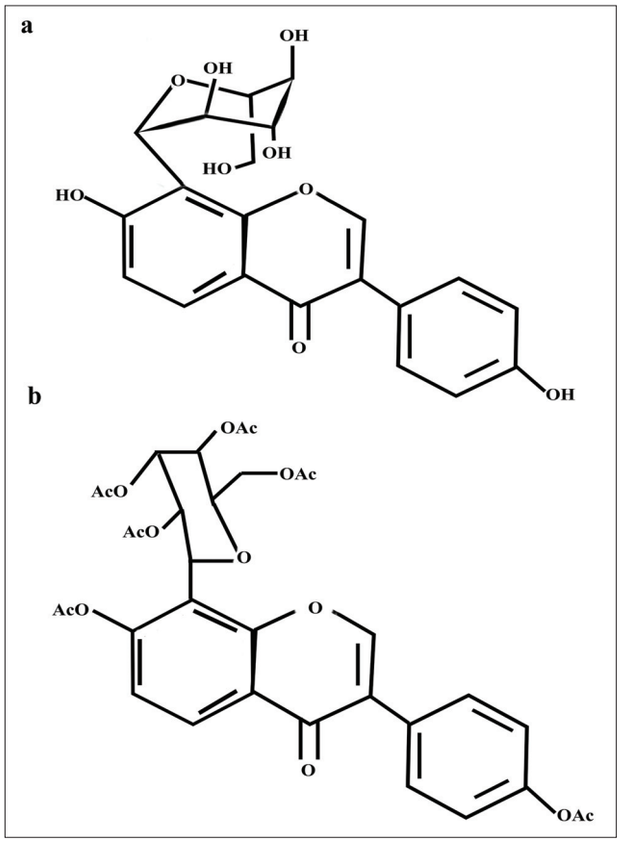

Fig. 1: Chemical structure of puerarin (a), acetylpuerarin (b).

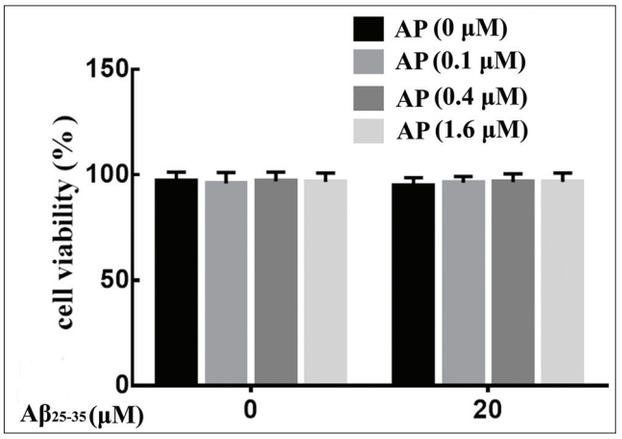

Fig. 2: Effect of acetylpuerarin on BV2 cell viability. The cells were treated with acetylpuerarin at different concentration for 1 h. After that, the cells were stimulated with $A\beta_{25-35}$ (20 μM) for another 12 h in the presence of acetylpuerarin. MTT assay was used to assess cell viability. The results were expressed as percentage of surviving cells in drug-treated groups over the cells in control group (only with culture medium). Each value indicated the mean±SD of three independent experiments. $P > 0.05$ when compared with control group.

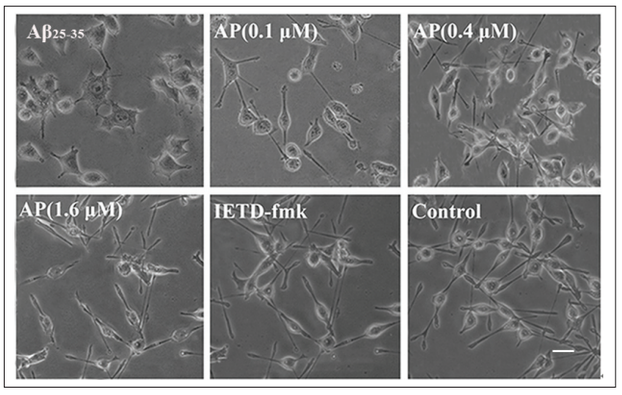

Fig. 3: Effect of acetylpuerarin on morphological changes of BV2 microglia cells. Phase contrast micrograph of BV2 microglia cells (20×) under different treatments. After stimulation by $A\beta_{25-35}$, the cells in model group showed the activated amoeboid phenotype. The statuses of cells pretreated with acetylpuerarin at different concentration were between model group and control group. The cells in caspase-8 inhibitor IETD-fmk group were close to the resting ramified cells in the control group. The cells in control group showed small round cytoplasm with an off-centered nucleus, resembling the resting ramified phenotype. The scale bar is 50 μm.

So this concentration range of acetylpuerarin was applied in all subsequent experiments and if acetylpuerarin had inhibitory effects on the $A\beta_{25-35}$-induced production of caspase-8, cleaved caspase-3 and inflammatory mediators, it was irrelevant for its cytotoxic action.

2.2. Effect of acetylpuerarin on morphological changes of BV-2 microglia cells

In order to observe the morphological changes of BV2 cells under activation by $A\beta_{25-35}$, the cells were cultured as described under "Experimental". Undisturbed microglia cells in the control group were uniform in size and displayed small round cytoplasm with an off-centered nucleus, resembling the resting ramified phenotype. After stimulated by $A\beta_{25-35}$ (model group), there was obvious expansion of cytoplasmic space with thickening and retraction filopodia, resembling the activated amoeboid phenotype and the cells assembled into groups. The status of cells pretreated with acetylpuerarin at different concentrations were between unstimulated ramified microglia in the control group and activated amoeboid microglia in the model group. The cells in caspase-8 inhibitor IETD-fmk group were close to the undisturbed microglia cells in the control group (Fig. 3).

2.3. Acetylpuerarin suppressed inflammatory cytokines IL-1β and TNF-α expression in Aβ25-35-induced BV2 microglia cells

To explore whether acetylpuerarin possessed anti-inflammatory effects against Aβ, we analyzed the expression of inflammatory cytokines in the $A\beta_{25-35}$-induced BV2 microglia cells. An ELISA assay was designed to measure the levels of IL-1β and TNF-α in culture supernatant. The results showed that $A\beta_{25-35}$ alone significantly induced IL-1β secretion (33.87±2.00 ng/ml, $p < 0.01$) versus the control group (5.48±0.56 ng/ml); but the increase was inhibited by acetylpuerarin in a dose-dependent manner (high-dose group, 15.43±1.22 ng/ml, $p < 0.01$; median-dose group, 19.73±0.86 ng/ml, $p < 0.01$; low-dose group, 25.47±0.74 ng/ml, $p < 0.05$) (Fig. 4a). Similarly, $A\beta_{25-35}$ increased the production of TNF-α (207.33±11.15 ng/ml, $p < 0.01$), versus the control group (27.40±1.8 ng/ml); and acetylpuerarin dose-dependently attenuated the change (high-dose group, 48.67±3.51 ng/ml, $p < 0.01$; median-dose group, 96.00±5.29 ng/ml, $p < 0.01$; low-dose group, 154.67±6.66 ng/ml, $p < 0.01$) (Fig. 4b).

The IL-1β and TNF-α mRNA and protein expression were detected using qRT-PCR and Western blot assay. The results showed that $A\beta_{25-35}$ caused more than a 5- and 2 -fold increase in the levels of IL-1β and TNF-α respectively ($p < 0.001$) versus the control group (Fig. 4c, e); and the application of acetylpuerarin reduced this increase in a dose-dependent manner ($p < 0.01$ or $p < 0.001$). At protein level (Fig. 4d, f), consistent variations were observed. IL-1β (0.856±1.016, $p < 0.001$) and TNF-α (1.022±0.0075, $p < 0.001$) increased markedly in $A\beta_{25-35}$ group versus the control group (IL-1β 0.0025±0.0007; TNF-α 0.008±0.002). However,

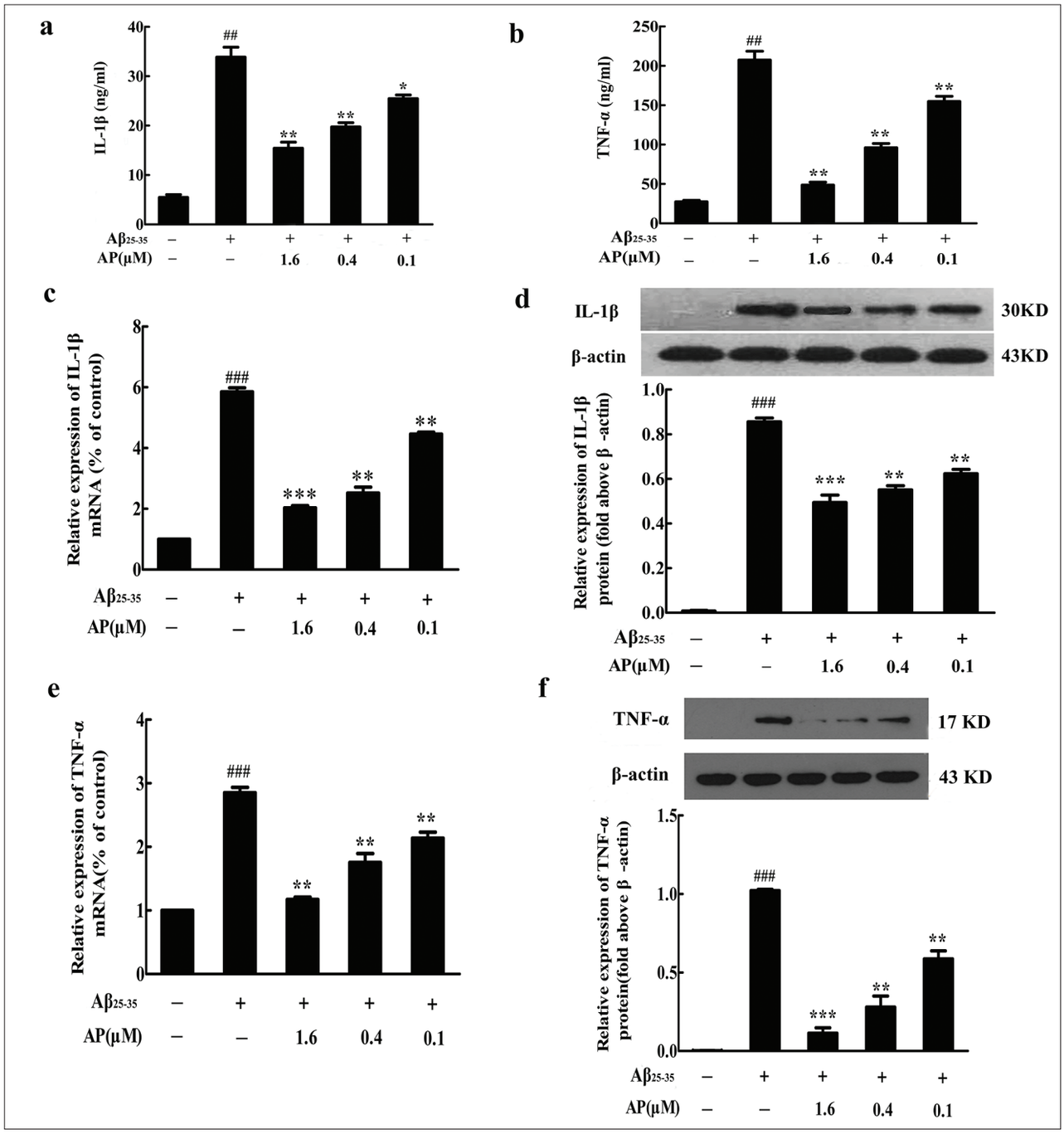

Fig. 4: Effect of acetylpuerarin on IL-1β and TNF-α expression in $A\beta_{25-35}$-stimulated BV2 microglia cells. BV2 cells were incubated and treated as described under "Experimental". The concentration of IL-1β and TNF-α in culture supernatant were measured by ELISA (a-b). The expressions of IL-1β and TNF-α mRNA were detected using quantitative real-time PCR (c, e). β-actin served as the reference gene. Fold changes were calculated with the $2^{-(\Delta\Delta Ct)}$ method. In histogram, the control group was standardized to one-fold. The expressions of IL-1β and TNF-α protein were detected using Western blot (d, f). Band intensities were quantified by Image-Pro Plus 6.0 and normalized to β-actin. Data represented as mean ± SD and pooled from three independent experiments. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus $A\beta_{25-35}$ group.

the pretreatment of acetylpuerarin dose-dependently inhibited the variations ($p < 0.01$ or $p < 0.001$).

2.4. Acetylpuerarin suppressed the expression of PKC-δ and IKKβ expression in Aβ25-35-induced BV2 microglia cells

To investigate whether the anti-inflammatory role of acetylpuerarin was associated with the modulation PKC-δ/IKKβ, the expression of PKC-δ and IKKβ in BV2 microglia cells were detected by qRT-PCR and Western blot. β-Actin was the internal control. The mRNA of PKC-δ (3.748±0.051-fold, $p < 0.001$), IKKβ (4.093±0.199-fold, $p < 0.001$) were distinctively higher in $A\beta_{25-35}$ group versus the control group; however, the levels of PKC-δ and IKKβ were decreased by acetylpuerarin in a dose-dependent manner ($p < 0.01$ or $p < 0.001$) (Fig. 5a). Similarly, at the protein level, PKC-δ (0.856±0.0389, $p < 0.001$) and IKKβ (0.367±0.0187, $p < 0.001$) were upregulated in $A\beta_{25-35}$ group versus control group (PKC-δ, 0.0027±0.0017; IKKβ, 0.002±0.001), and acetylpuerarin reduced the levels in a concentration-dependent manner ($p < 0.05$ or $p < 0.01$ or $p < 0.001$)

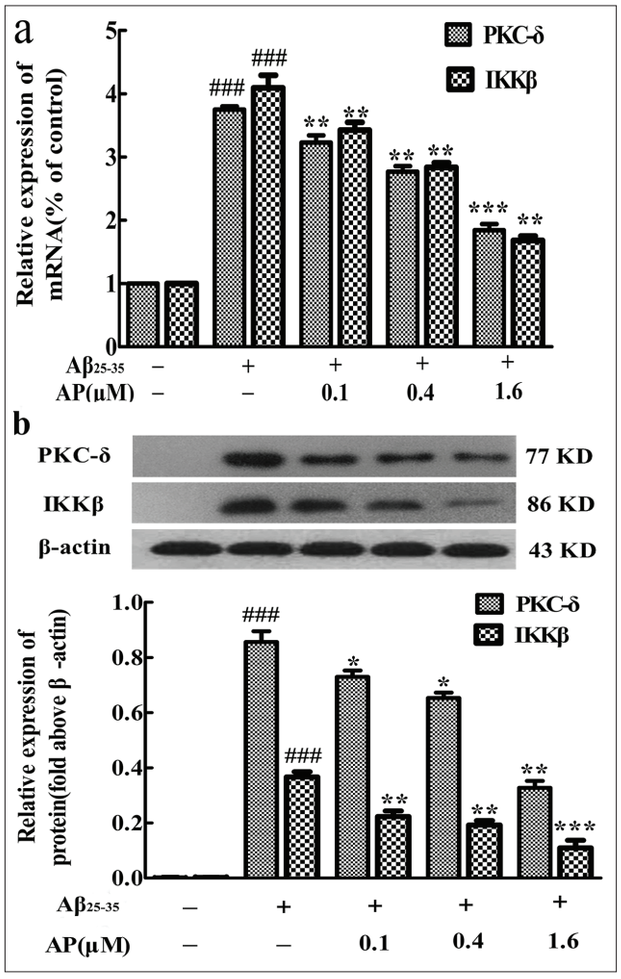

Fig. 5: Effect of acetylpuerarin on PKC-δ and IKKβ expression in $A\beta_{25-35}$-induced BV2 microglia cells. BV2 cells were pretreated with the indicated concentrations of acetylpuerarin 1 h before $A\beta_{25-35}$ treatment for another 12 h. The PKC-δ and IKKβ mRNA expressions were detected using (a) Quantitative real-time PCR. β-actin served as the reference gene. In the histogram, the control group was standardized to one-fold. The PKC-δ and IKKβ protein expressions were detected using (b) Western blot. β-Actin was used as internal standard. Results were mean±SD from three independent experiments. $^{###}P < 0.001$, compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with $A\beta_{25-35}$ group.

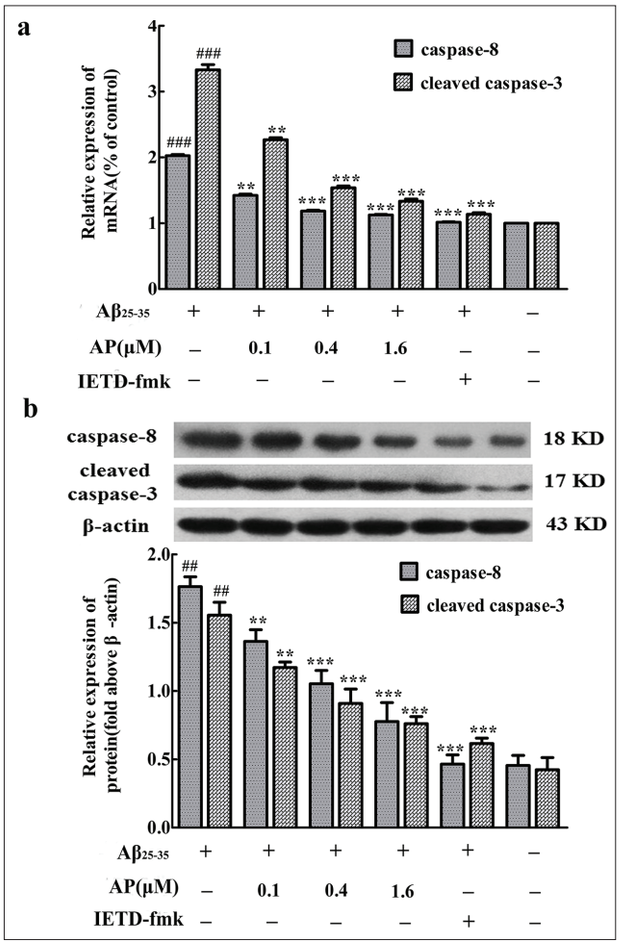

Fig. 6 a-b: Effect of acetylpuerarin on caspase-8, cleaved caspase-3 and iNOS activation in $A\beta_{25-35}$-stimulated BV2 microglia cells. The cells were treated with acetylpuerarin at different concentration for 1 h. After that, the cells were stimulated with $A\beta_{25-35}$ (20 μM) for another 24 h in the presence of acetylpuerarin. The activation of caspase-8 and cleaved caspase-3 were measured by qRT-PCR (a) and Western blot (b).

(Fig. 5b). The finding was in agreement with our previous in vivo study (Meng et al. 2013).

2.5. Acetylpuerarin suppressed the activation of caspase-8/caspase-3 in Aβ25-35-induced BV2 microglia cells

To detect whether the anti-inflammatory role of acetylpuerarin was associated with the regulation of PKC-δ upstream molecules caspase-8 and caspase-3, the activity of caspases-8, cleaved caspase-3 and caspase-downstream protein iNOS were examined by qRT-PCR, immunofluorescence and Western blot analysis in $A\beta_{25-35}$-stimulate BV2 microglia cells. As shown in Fig. 6a-b, the activation of caspases-8 and cleaved caspase-3 was notablely enhanced by $A\beta_{25-35}$ ($p < 0.01$, versus the control group); while the pretreatment of acetylpuerar in dose-dependently attenuated the activation ($p < 0.01$ or $p < 0.01$); and caspases-8 inhibitor IETD-fmk inhibited the activation significantly ($p < 0.001$). Furthermore, the expression of cleaved caspase-3 and iNOS was detected using immunofluorescence (Fig. 6c-d). It showed that the positive material of cleaved caspase-3 and iNOS immunoreactive were red, localized in the cytoplasm, and the intensity of expression was different. The most cleaved caspase-3 and iNOS positive cells were found in the model group, with the deepest coloring; the positive cells in control group were the least, with the lightest coloring; the cells pretreated with acetylpuerarin at different concentrations were between the control and the model group; and the positive cells in caspase-8 inhibitor IETD-fmk group were close to the control group.

2.6. Acetylpuerarin suppressed the expression of IL-6 and IL-1β in Aβ1-42-treated rats

To find out whether acetylpuerarin possessed the same effects in AD model rats as in vitro, we examined the levels of IL-6 and IL-1β in $A\beta_{1-42}$-treated rats using ELISA, immunohistochemistry and Western blot analysis. The level of IL-6 in serum of rats was detected by the ELISA method. Results showed that injection of $A\beta_{1-42}$ remarkably increased the level of IL-6 in serum (1052.61±27.10 pg/ml, $p < 0.001$) versus control group (205.52±6.24 pg/ml); after acetylpuerarin treatment, the level of serum IL-6 decreased and in a dose-dependent manner (low dose group, 273.21±31.12 pg/ml, $p < 0.001$; high dose group, 209.27±5.29 pg/ml, $p < 0.001$, versus model group) (Fig. 7a). In immunohistochemistry, the staining results of IL-1β showed that the positive material of immunoreactive were brown, localized in the cell membrane and cytoplasm. Among them, there were the most IL-1β positive cells in the model group, with the deepest coloring, and the most positive granular pattern (Fig. 7b). Quantitative results showed: the number of IL-1β hippocampal positive cells in model group (40±3.20) was more than that in control

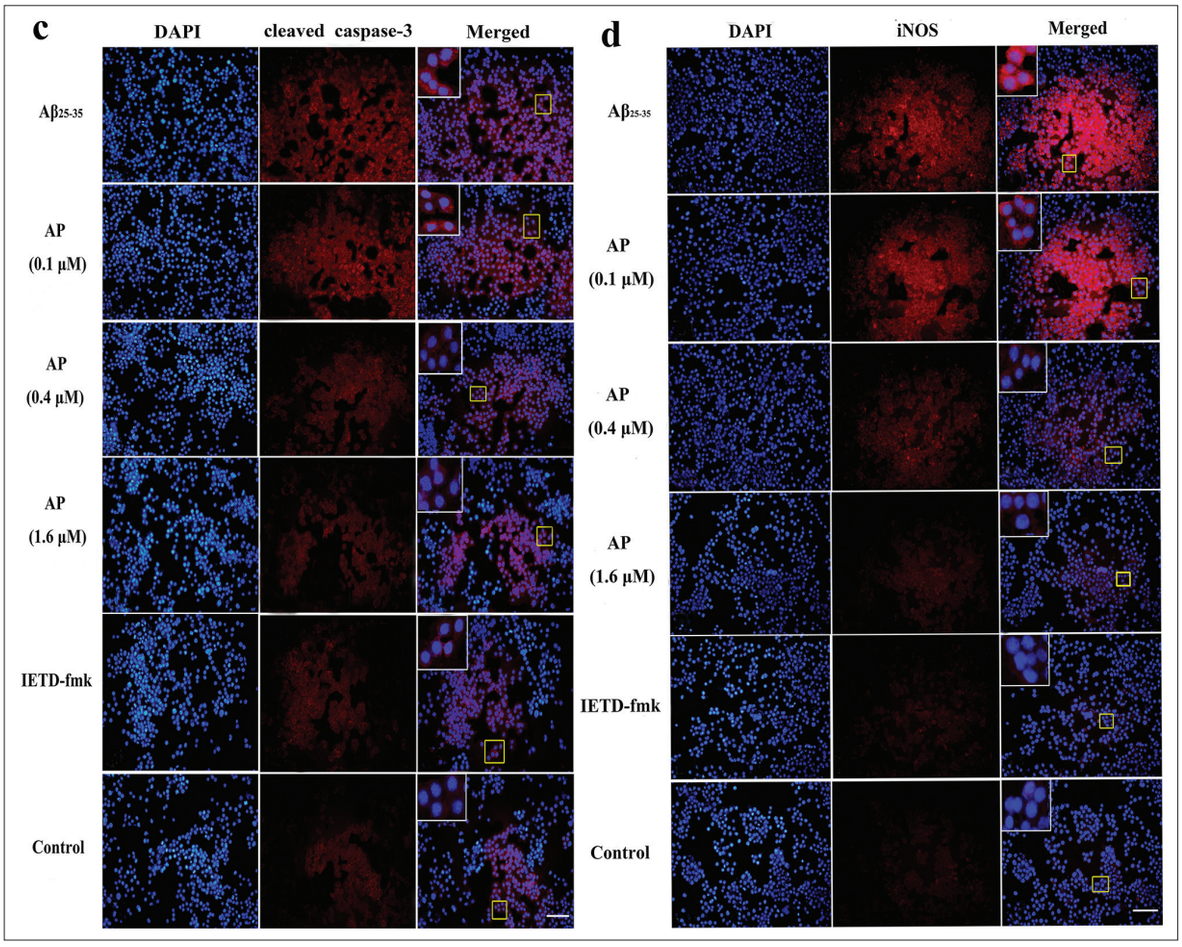

Fig. 6 c-d: β-Actin was used as internal control. Representative images of caspase-3-positive and iNOS-positive BV2 microglia cells were taken after immunofluorescent staining (c-d). Cells were stained with DAPI for visualization of nuclei (blue); caspase-3 and iNOS immune positive reaction were stained red. The scale bar is 50 μm. Data were shown as mean ± SD from three independent experiments. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the control group; $^{**}P < 0.01$, $^{***}P < 0.001$, compared with $A\beta_{25\text{-}35}$ group.

group (8±1.80, $p < 0.01$), acetylpuerarin low-dose group (32±2.00, $p < 0.05$) and acetylpuerarin high-dose group (20±1.60, $p < 0.01$). This indicated that the level of IL-1β in the hippocampus increased after $A\beta_{1\text{-}42}$ injection, and acetylpuerarin treatment suppressed the elevations (Fig. 7c). Western blot method was used to detect the quantitative expression of IL-1β in hippocampal tissue. The results showed that bilateral hippocampal injection of $A\beta_{1\text{-}42}$ could significantly increase the expression of IL-1β, about three times as much as in the control group. Acetylpuerarin dose-dependently reduced the expression of IL-1β induced by $A\beta_{1\text{-}42}$ (low-dose group, $P < 0.05$; high-dose group, $P < 0.001$) (Fig. 7d).

3. Discussion

Aβ, a polypeptide containing 39-43 amino acids, is derived from amyloid precursor protein (APP). It's physiological concentrations have trophic effects on the nerve, but when Aβ production and clearance loses dynamic equilibrium, with increasing concentration, it will gather and form the senile plaques and produce nerve toxic effects, causing abnormal phosphorylation of tau protein, synaptic changes, loss of neurotransmitters, oxidative stress, glial cell proliferation and inflammation, neuronal apoptosis, and ultimately lead to the occurrence of Alzheimer disease (AD) (Heneka et al. 2015; Heppner et al. 2015). $A\beta_{25\text{-}35}$ is an active center of Aβ (Pike et al. 1993), both $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$ can be used to establish cell damage model (Yu et al. 2013) and the animal model of AD (Chen et al. 2015; McLarnon and Ryu 2008; Sachdeva and Chopra 2015) to test some drug's anti-inflammation effects.

Microglia cells, the intrinsic macrophages of the central nervous system (CNS), play an important role in immune defense and inflammatory response. The studies on microglia suggest that a series of stimuli, such as Aβ, oxygen free radicals can trigger the activation of microglia. Activated microglia would suffer visible morphological changes, including soma growth, decreased branching, and the acquisition of an amoeboid form (Chuang et al. 2015). On the other hand, the activated microglia cells produce a wide spectrum of pro-inflammatory mediators (IL-1β, IL-6, TNF-α, iNOS, et al), which cause neuronal cell damage and ultimately lead to neuronal cell death. This is a common characteristic found in AD. In this study, we found both $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$ induced the activation of microglia. In the in vitro study, after stimulated by $A\beta_{25\text{-}35}$, a typical morphological change associated with activation was evident in BV2 microglia cells (resting ramified phenotype to activated amoeboid phenotype). Morever, the expressions of pro-inflammatory cytokines IL-1β, IL-6 were significantly increased in vitro and in vivo. So, both the BV2 microglia cells and Wistar rats were successfully activated by $A\beta_{25\text{-}35}$ or $A\beta_{1\text{-}42}$ to establish the in vitro and in vivo inflammatory model. Pretreatment with acetylpuerarin at different concentrations alleviated the morphological changes and the expressions of pro-inflammatory cytokines. Therefore, inhibition of pro-inflammatory cytokines by acetylpuerarin, as shown in this study, could be beneficial in the treatment of AD.

Caspase-3 is regarded as a key executor of the apoptotic cell death program within the family of cysteinyl aspartate-specific proteases. Nevertheless, recent studies have demonstrated its non-apoptotic functions, such as regulation of synaptic plasticity

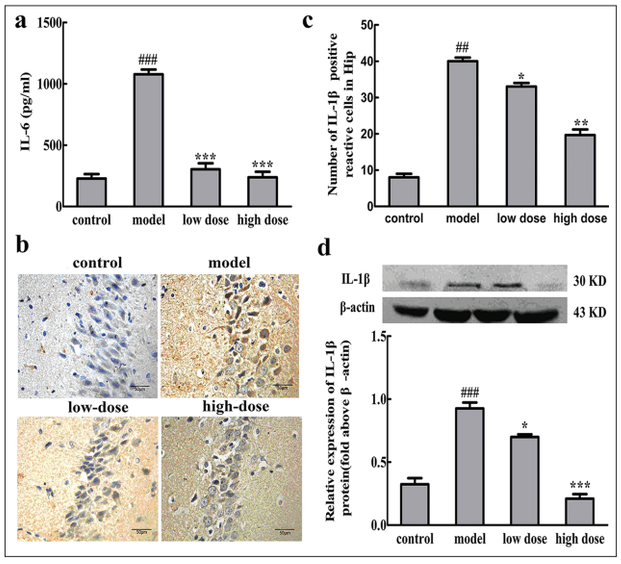

Fig. 7: Effect of acetylpuerarin on IL-6 and IL-1β expression in hippocampus of $A\beta_{1-42}$-treated rats. Serum specimen and brain sample were prepared as described under "Experimental". The level of IL-6 in serum was detected by ELISA method (a). The expression of IL-1β in the hippocampus was detected using (b-c) Immunohistochemistry and (d) Western blot. b: Representative immunohistochemistry for IL-1β in hippocampus (40×). The scale bar is 50 μm. c: Quantitative assessment of IL-1β-positive reactive cells in hippocampus. d: Representative western blots and quantitation of IL-1β. β-Actin was used as internal control. Data were shown as mean ± SD and collected from three independent experiments with 5 rats per experiment, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with model group.

and function, anti-inflammation (Venero et al. 2013). It was found that stimulation of microglia with different proinflammogens activate caspase-8 and caspase-3/7 (so-called apoptotic caspases) in microglia without triggering cell death in vitro and in vivo, and the orderly activation of caspase-8 and caspase-3/7 governs microglia activation in a PKC-δ-dependent manner. Inhibition of the caspase-8 and caspase-3 pathway could effectively block microglia activation and neuron death and exert neuro-protective effects (Burguillos et al. 2011). In this in vitro study, we found that the mRNA and protein expressions of caspase-8 and cleaved caspase-3 in the $A\beta_{25-35}$ group were significantly upregulated, which indicated that caspase-8 and caspase-3 were involved in microglia activation induced by $A\beta_{25-35}$. The expressions of caspase-8 and cleaved caspase-3 in the caspase-8 inhibitor IETD-fmk group were significantly decreased. These results indicated that caspase-3 is in the downstream of caspase-8, and is activated by caspase-8, the same result as previous study (Burguillos et al. 2011). Acetylpuerarin markedly inhibited the expressions of caspase-8 and cleaved caspase-3 and in a concentration-dependent manner. Taken together, we consider that acetylpuerarin possibly inhibit microglial activation by suppressing caspase-8 and caspase-3 activity.

Protein kinase C (PKC), a family of serine/threonine kinases with different isoforms, is a pivotal signaling enzyme which regulates various cellular functions (Bhatt et al. 2010). It has been reported that PKC plays an important role in microglial activation (Nakai et al. 1998). PKC-δ, one of the PKC isoforms, can be cleaved into a 40-kDa active fragment by caspases and its activation can be reduced by caspase-3 inhibitor DEVD-fmk or selective knockdown siRNA of caspase-3, which shows that PKC-δ activation is rely on caspase activity (Burguillos et al. 2011). Furthermore, it has been demonstrated that PKC-δ can regulate NF-κB activation through IKK complexes and phosphorylation of the NF-κB inhibitor IkB (Storz et al. 2004; Vancurova et al. 2001). Activation of NF-κB is the key event for the induction of all major pro-inflammatory mediators. In resting status, NF-κB is associated with the inhibitory protein IκBα as an inactive form in the cytoplasm. An extracellular stimulus can induce the phosphorylation of IκBα mediated by the IκBα kinase (IKK), mainly including IKKα and IKKβ, after IκBα degradation, the active complex of NF-κB translocates into nucleus from cytoplasm and stimulates the inflammation-related genes including IL-1β, TNF-α, iNOS, cyclooxygenase-2 (COX-2) and so on (Hayden and Ghosh 2008; Karin and Ben-Neriah 2000; Li and Verma 2002). Taken together, PKC-δ, IKKβ, NF-kB are all important participators in the development of inflammation. Here, the mRNA and protein levels of PKC-δ, IKKβ were increased in vitro after stimulation by $A\beta_{25-35}$. These results indicated that PKC-δ is an important promoter in the activation of the IKKβ/NF-kB pathway. However, acetylpuerarin concentration-dependently weakened the effect. This result was the same as in previous in vivo study (Meng et al. 2013).

IL-1β is a typical pro-inflammatory cytokine which can cause inflammatory cascades in vitro and in vivo. It has been shown that IL-1β can promote β-secretase cleavage of APP in human astrocytes and thereby increase $A\beta_{1-40}$ and $A\beta_{1-42}$ production (Blasko et al. 2000). It is also known that accumulation of plaques and the formation of neurofibrillary tangles are correlated with increased IL-1 levels in the AD brain (Griffin 2006; Hein et al. 2012). In this study, we found that the expression of IL-1β was increased after Aβ-stimulation in vivo and in vitro, and acetylpuerarin suppressed the increase. Besides IL-1β, hypernomic production of TNF-α from the activated microglia devotes to uncontrolled inflammation in neurodegenerative disorders (Shi et al. 2016). The same result was observed in our in vitro study.

IL-6 is a multi-functional cytokine which can play a mediated role in the acute inflammation phase and has significant effect on neural development, differentiation, regeneration, neurodegeneration. It has been proved to be linked to brain memory (Campbell 1998). Some studies showed that serum IL-6 and C-reactive protein (CRP) were prospectively related to cognitive decline in well functioning elders (Yaffe et al. 2003); high levels of α1-antichymotrypsin (ACT), IL-6 and CRP in plasma were associated with an increased risk of AD respectively (Engelhart et al. 2004). It is found that there is a close relationship between IL-6 and neurofibrillary tangles (Weisman et al. 2006). Luterman et al. (2000) confirmed that IL-6 levels in peripheral blood, cerebrospinal fluid and brain tissue of AD patients were significantly increased. Hull's study (Hull et al. 1996) showed that during the early stage of formation of senile plaques in AD, there was a large amount of IL-6 in senile plaques of AD patients, and no expression of IL-6 in non AD patients. In this in vivo study, we found that the level of serum IL-6 in rat was elevated after $A\beta_{1-42}$ treatment, and acetylpuerarin dose-dependently suppressed the elevation.

iNOS is a responsive enzyme in inflammation; its upregulation is not only a proliferation marker of responsive glial, but is also related to neuronal damage. iNOS can continuously catalyze NO production, which can cause neurotoxicity and lead to neuron necrosis or apoptosis (Haas et al. 2002; Steinert et al. 2010). Zhu and Qian (2006) have demonstrated that reduced learning and memory in rats is related to neurotoxicity induced by NOS overexpression. Drugs that suppress nNOS and iNOS activity or expression can protect neurons and improve learning and memory in rats. In the present study, we found that the expression of iNOS was increased after $A\beta_{25-35}$ stimulation and the variation was ameliorated by acetylpuerarin in a concentration-dependent manner. The result is the same as in our previous in vivo study (Meng et al. 2013).

Puerarin is an isoflavanone glycoside extracted from Radix puerariae. It has been demonstrated to possess a wide spectrum of pharmacological effects such as vasodilation, cardio-protection, anti-oxidant, anti-cancer, neuro-protection, anti-inflammation, etc (Zou et al. 2013). Studies reported that puerarin pretreatment blocked the activation of the inflammatory biomarkers iNOS, TNF-α, and NF-κB induced by 3-nitropropionic-acid in rats's striata, hippocampus, and cortices (Mahdy et al. 2014). But the poor solubility, short elimination half-life, and low oral bioavailability limit its clinical application. Acetylpuerarin is a new type of isoflavonoid created by introducing a latent chain into puerarin. This modification increases liposolubility and allows it to cross the BBB easily, which means a better use in the future. In the present study, acetylpuerarin notably decreased the expressions of caspase-8, cleaved caspase-3, PKC-δ, IKKβ, and the

downstream pro-inflammatory mediators, IL-1β, IL-6, TNF-α and iNOS in vitro and in vivo. These results indicate that acetylpuerarin may protect microglia cells against Aβ-induced inflammation via the PKC-δ-dependent caspase signaling pathway. So we believe that acetylpuerarin may play a protective role in Alzheimer's disease, and it also may be a novel preventive or therapeutic approach for the devastating disease.

4. Experimental

4.1. Reagents and drugs

Acetylpuerarin was obtained from the Shandong Academy of Medical Science (MW: 668D. Lot: 2002-12-2211). $A\beta_{25-35}$ and $A\beta_{1-42}$ were purchased from Sigma-Aldrich (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) and other reagents for cell culture were purchased from Gibco (USA). The anti-PKC-δ (ab47473) antibody was purchased from Abcam (USA); anti-caspase-8p18 (sc-7890) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA); anti-cleaved caspase-3 (9661s) and anti-iNOS (13120s) antibodies were purchased from Cell Signaling Technology (USA); anti-TNF-α (BS1857), anti-IL-1β (BS3506) and anti-IKKβ (BS1407) antibodies were purchased from Bioworld Technology (USA). Tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary antibody (ZF-0316) was purchased from Zhongshan Jinqiao Biological Engineering Co. (Beijing, China). Trizol (9108), Primer Script™ RT Reagents Kit with gDNA Eraser (RR047A) and SYBR® Premix Ex Taq™ PCR amplification kit (RR420A) were purchased from Takara biotechnology Co., Ltd, (Dalian, China). Streptavidin-biotin complex and diaminobenzidine (DAB) staining kits were purchased from Zhongshan Jinqiao Biological Engineering Co. (Beijing, China). A Cytoplasmic Protein Extraction kit and a BCA protein assay kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

4.2. Cells and animals

The mouse BV2 microglial cell line (3111C0001CCC0063) was purchased from Peking Union Medical College of Preclinical Medicine (Beijing, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in a incubator with 5% CO_2 at 37°C.
Seven-month-old female Wistar rats (350-380 g) were supplied by the Animal Center of Lukang Biotechnology Ltd. (Ji ning, Shandong, China; SPF Grade II). Rats were housed in a climate-controlled room (temperature, 23±1 °C, 50±2 % humidity) under a 12 h light/12 h dark cycle, with standard animal food and water *ad libitum*. All animal experiments complied with the US National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Care and Use Committee of Weifang Medical University.

4.3. Treatment of acetylpuerarin, Aβ1-42 and Aβ25-35

Acetylpuerarin was dissolved in DMSO, diluted in serum-free culture medium to the desired concentration when experimented, and the final concentration of DMSO was less than 0.1%. $A\beta_{25-35}$ and $A\beta_{1-42}$ were dissolved in sterile double distilled water at a concentration of 5 μg/μl, incubated for 7 days and 72 h respectively in an incubator at 37 °C to aggregate into fibrils-like structures, oligomers and then stored at -20 °C. When in use, the stock solution was further diluted to desired concentrations with culture medium.

4.4. Experimental design

BV2 microglia cells were seeded in 96-well plates at a density of 1×10^4 cells/ml and were randomly divided into six groups: (1) control group (culture medium); (2) $A\beta_{25-35}$ group ($A\beta_{25-35}$ 20 μM); (3) low-dose group ($A\beta_{25-35}$ 20 μM plus 0.1 μM acetylpuerarin); (4) median-dose group ($A\beta_{25-35}$ 20 μM plus 0.4 μM acetylpuerarin); (5) high-dose group ($A\beta_{25-35}$ 20 μM plus 1.6 μM acetylpuerarin); and (6) caspase-8 inhibitor (IETD-fmk) group ($A\beta_{25-35}$ 20 μM plus 20 μM IETD-fmk). For acetylpuerarin treatment groups: pretreated with different concentrations of acetylpuerarin for 1 h, and then were incubated with $A\beta_{25-35}$ (20 μM) for another 12 h. The caspase-8 inhibitor group was pretreated with caspase-8 inhibitor (IETD-fmk) for 1 h, and then were incubated with $A\beta_{25-35}$ (20 μM) for another 24 h. Cells with different treatments were used for subsequent MTT assay, morphological detection, ELISA assay, quantitative real-time PCR, immunofluorescence and Western blot analysis. These experimental conditions were kept constant in all performed tests in this study.
The rats (n= 40) were divided into four different groups (10 rats in each group) at random: (1) sham surgery control group (rats were injected intrahippocampal with sterile normal saline); (2) model group (5 μg $A\beta_{1-42}$ intrahippocampal injection); (3) low-dose acetylpuerarin group ($A\beta_{1-42}$ intrahippocampal injection plus 100 mg/kg acetylpuerarin); and (4) high-dose acetylpuerarin group ($A\beta_{1-42}$ intrahippocampal injection plus 200 mg/kg acetylpuerarin). For the drug treatment group rats, acetylpuerarin (100, 200 mg/kg) was administered intragastricly once daily for 12 days.

4.5. AD model rats establishment

AD model rats were established with the method of bilateral intrahippocampal amyloid-β $(A\beta)_{1-42}$ injections as previously described (Meng et al. 2013).

4.6. Serum specimen preparation

After disinfection of the precordial skin of rats, 4 ml of heart blood was extracted using a 5 ml sterile syringe and stood for 30 min. The blood was centrifugted for 15 min at a speed of 3000 rmp/min at 4 °C to separate serum. The serum was stored at -80 °C until detected.

4.7. Brain sample preparation

Brain sample was prepared with our previously described method (Meng et al. 2013).

4.8. Cell viability assay

MTT assay was used to assess cell viability. Briefly, BV2 microglia cells (1×10^4 cells/ml) were seeded into 96-well plates and pretreated with acetylpuerarin at different concentrations (0.1, 0.4, and 1.6 μM) for 1 h. After that, the cells were stimulated by $A\beta_{25-35}$ (20 μM) which could induce inflammation in BV2 cells in previous report (Yu et al. 2013) for an additional 12 h in the presence of acetylpuerarin. Then medium was removed and new FBS-free medium containing 0.5 mg/ml MTT was added and cells were incubated at 37 °C for 4 h. The supernatants were then removed and DMSO (200 μl/well) was added. Shook the plates for 10 min to solubilize formation of formazan. The absorbance of each well was measured with a microplate reader at 570 nm wavelength.

4.9. BV-2 microglia cells morphological detection

The changes of BV-2 microglia cellular morphology were assessed using inverted phase contrast microscope and the pictures of each group were taken (20×).

4.10. Enzyme-linked immunosorbent assay (ELISA)

The method of enzyme linked immunosorbent assay (Elisa) was used to detect the level of IL-1β, TNF-α in culture supernatant of BV2 microglia cells and IL-6 in serum of rats, and the relevant procedures were strictly in accordance with the manufacturer's instructions. A standard curve was established according to the concentration of standard pipes and the optical density (OD) value, and then calculated the linear regression equation. Finally the concentration of IL-1β, TNF-α and IL-6 were calculated according to the linear regression equation and sample holes' OD values.

4.11. Quantitative real-time PCR

Quantitative real-time PCR analysis was conducted to measure the expression of mRNA. Total RNA was isolated from BV2 microglia cells with Trizol reagent and the RNA purity was measured using spectrophotometry. cDNA was synthesized from 1 μg of total RNA using a Primer Script™ RT Reagent Kit in 20 μl reaction volume and the procedures were strictly in according with the manufacturer's instructions. Single stranded cDNA was amplified according to the directions of SYBR® Premix Ex Taq™ PCR amplification kit in an iQ-5 qPCR (Bio-Rad Co., Ltd, CA, USA) thermal cycler. The PCR cycling program was set for pre-denaturation at 95 °C for 30 s, followed by denaturation at 95 °C for 5 s, annealing at 57 °C (for PKC-δ, IKKβ and IL-1β)/60 °C (for caspase-8, caspase-3 and TNF-α) for 30 s, extension at 72 °C for 30 s. Primers used in the present study were designed and synthesized by Takara Biotechnology Co., Ltd, (Dalian, China) and the primers specificity were validated utilizing NCBI primer Blast (Table). β-Actin served as the reference gene and was used for sample normalization. Threshold cycle (Ct) values were used to calculate fold changes according to the $2^{-(\Delta\Delta Ct)}$ method.

4.12. Western blot analysis

Total protein was isolated from the BV2 microglia cells and tissues. The concentration of each sample was then determined using a BCA protein assay reagent kit. After that, each sample was heated at 100 °C for 10 min with loading buffer. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%SDS-PAGE) and electro-transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were soaked in 5% nonfat dry milk (blocking buffer) at room temperature for 2 h and incubated with primary antibodies overnight at 4 °C. The primary antibodies were rabbit polyclonal antibody against caspase-8 (1:1000), cleaved caspase-3 (1:500), PKC-δ (1:1000), IKKβ (1:500), IL-1β (1:500), TNF-α (1:500) and mouse monoclonal antibody against β-actin (1:1000). On the second day, the membranes were washed thrice with TBST and then incubated with secondary antibodies (goat anti-rabbit IgG conjugated to HRP, 1:2500, goat anti-mouse IgG conjugated to HRP, 1:8000) for 1 h on a shaker at room temperature. After that, the membranes were washed three times with TBST, and immune complexes were visualized with chemiluminescence (ECL) reagent according to instructions. For quantification, protein blots were scanned, and the band intensities were measured by the Image-Pro Plus 6.0 image analysis software and normalized against β-actin.

4.13. Immunofluorescence and immunohistochemistry analyses

For immunofluorescence analyses, cells were subcultured into poly-L-lysine-coated coverslips. After indicated drug treatments, BV2 microglia cells were fixed with 4% paraformaldehyde for 30 min, treated with 0.1% TritonX-100 for 10 min and blocked with 10% normal goat serum at room temperature for 30 min. Then the cells were incubated with primary antibody (rabbit polyclonal antibody against cleaved caspase-3, 1:200; rabbit monoclonal antibody against iNOS, 1:200) at 4 °C overnight. The next day, the cells were washed with PBS solution for three times and incubated with TRITC-conjugated goat anti-rabbit secondary antibody (1:500) at 37 °C for 2 h. Nuclear was stained with DAPI for 10 min. Images were collected using a fluo-

rescence microscope. The condition of each photo shoot was consistent. Acquired images were analyzed using the Image-Pro Plus 6.0 image analysis software.
The hippocampus was cut into coronally 5 μm-thick sections. Immunohistochemistry was performed according to our previously described method (Meng et al. 2013).

4.14. Statistical analysis

Data were collected from at least three independent experiments and expressed as mean±SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test with SPSS sofeware (version 17.0). Statistical significance was considered as $P < 0.05$.

Table: Primer sequences for qRT-PCR

Target mRNA	Forward(F) and reverse(R) Primers(5'-3')
caspase-8	F: GCTGCCCTCAAGTTCCTGT
	R: GATTGCCTTCCTCCAACATC
cleaved caspase-3	F: GAGCCAGAGCAGAGACTTGG
	R: CATCATCCACACAAACCAGAA
PKC-δ	F: CAGACCAAGGACCACCTGTT
	R: GCATAAAACGTAGCCCGGTA
IKKβ	F: AAGTCCGAGGTCCCGTGAGT
	R: GCTTCGGCCACCAGTTCTT
IL-1β	F:ACCACTGCTCAGGTCCACTGTC
	R: GCTGTCACTATCCCGGAGTTCA
TNF-α	F: TGTCCCAGCATCTTGTGTTTCT
	R: CCCTTTACTCTGACCCCTTTATTGT TTCCCTT-TACTCTGACCCCTTTATTGT CCCTTTACTCT-GACCCCTTTATTGT
β-actin	F: GTGCTATGTTGCTCTAGACTTCG
	R: ATGCCACAGGATTCCATACC

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