

Full Paper

Xanthoceraside Inhibits Pro-inflammatory Cytokine Expression in $A\beta_{25-35}$ /IFN- γ -Stimulated Microglia Through the TLR2 Receptor, MyD88, Nuclear Factor- κ B, and Mitogen-Activated Protein Kinase Signaling PathwaysYue Qi^{1,2}, Li-Bo Zou^{1,*}, Li-Hua Wang³, Ge Jin¹, Jin-Jin Pan¹, Tian-Yan Chi¹, and Xue-Fei Ji¹¹Department of Pharmacology, Life Science and Biopharmaceutics School, Shenyang Pharmaceutical University, Shenyang 110016, PR China²Department of Pharmacology, The Second Hospital Affiliated to Liaoning Chinese Medical University, Shenyang 110034, PR China³Shenyang Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, PR China

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Abstract. An accumulating body of evidence suggests that Alzheimer's disease (AD) is associated with microglia-mediated neuroinflammation and pro-inflammatory cytokine expression. Therefore, the suppression of neuroinflammation and pro-inflammatory cytokine might theoretically slow down the progression of AD. Xanthoceraside, a novel triterpenoid saponin extracted from the husks of *Xanthoceras sorbifolia* BUNGE, has potent anti-inflammatory and neuroprotective effects. However, the molecular mechanism underlying its anti-inflammatory action remains unclear. In the present study, we attempted to determine the effects of xanthoceraside on the production of pro-inflammatory mediators in amyloid β_{25-35} ($A\beta_{25-35}$) / interferon- γ (IFN- γ)-stimulated microglia. Our results indicated that xanthoceraside (0.01 and 0.1 μ M) significantly inhibited the release of nitric oxide (NO) and pro-inflammatory cytokines interleukin-1 β and tumor necrosis factor- α in a concentration-dependent manner. Reverse transcriptase–polymerase chain reaction and western blotting analyses showed that xanthoceraside decreased the $A\beta_{25-35}$ /IFN- γ -induced production of cyclooxygenase-2 and inducible NO synthase. These effects were accompanied by inhibited activities of nuclear factor- κ B and mitogen-activated protein kinase through Toll-like receptor 2 in a myeloid differentiation protein 88-dependent manner. Our results provide support for the therapeutic potential of xanthoceraside in AD.

Keywords: xanthoceraside, cytokine, microglia, nuclear factor- κ B, mitogen-activated protein kinase

Introduction

Alzheimer's disease (AD) is a neurological disorder characterized by memory impairment and progressive dementia. Extracellular β -amyloid deposits are assumed to be pathological hallmarks of AD (1). These deposits form senile plaques and intracellular neurofibrillary tangles made up of the microtubule-associated protein

tau in the brain parenchyma involved in cognitive functions. Given that clusters of activated microglia located in amyloid plaques in the brains of AD patients (2), extensive neuropathological and neuroradiological studies have investigated the association between neuroinflammation and AD. Several studies also documented that neuroinflammation has a pivotal role in the onset and progression of AD (3 – 5). Epidemiological and clinical studies found that anti-inflammatory therapies delay dementia onset, slow down AD progression, and reduce the severity of cognitive AD symptoms (6, 7). Microglia-associated neuroinflammation contributes to several

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potential damages that eventually lead to synaptic dysfunction, synaptic loss, and neuronal death (6, 8).

Microglia, the resident macrophages of the central nervous system (CNS), are exquisitely sensitive to brain injuries and diseases. They serve as the first line of defense in the brain to protect the CNS from insults. Upon stimulation or neuronal injury, microglia are rapidly activated in response to several pathological pathways, including lipopolysaccharide (LPS), amyloid protein, and interferon- γ (IFN- γ) (9, 10). Notably, microglia exposed to these stimulated factors show altered morphologies and phenotypes and release neuro-inflammatory molecules such as nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor- α (TNF- α), and interleukin (IL)-1 β , all of which contribute to neuronal dysfunction and cell death in a vicious cycle (11, 12). Thus, regulating microglial activation might be a potential therapeutic approach in AD.

Xanthoceraside (Fig. 1A), a novel triterpenoid saponin extracted from the husks of *Xanthoceras sorbifolia* BUNGE (XB), is commonly used as traditional medicine for the treatment of rheumatism in China. Experimental data indicated that XB husk extract significantly improves the learning and memory dysfunction in rats after bilateral occlusion of the common carotid arteries (13). Xanthoceraside could also alleviate the amyloid β_{25-35} ($A\beta_{25-35}$)-induced impairment of spatial memory and oxidative stress in mice (14). Our previous study also demonstrated the anti-inflammatory effects of xanthoceraside in mice by down-regulating the expression of inducible NO synthase (iNOS) and nitrotyrosine levels in the hippocampus and by up-regulating IL-4 mRNA expression (15). However, the molecular mechanism underlying its anti-inflammatory action remains unclear.

In this study, we investigated the effects of xanthoceraside on pro-inflammatory cytokines to elucidate its potential anti-inflammatory mechanisms through the pathway of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and P38 signaling pathways, in $A\beta_{25-35}$ /IFN- γ -stimulated microglia.

Materials and Methods

Materials

Xanthoceraside ($\geq 99\%$ purity) was provided by the Shenyang Institute of Applied Ecology, Chinese Academy of Sciences (Shenyang, China) (16), and its chemical structure is shown in Fig. 1A. Xanthoceraside was dissolved in dimethyl sulfoxide (DMSO) and Dulbecco's modified Eagle's medium (DMEM) or minimum essential medium (MEM) at a concentration of 1 mM and

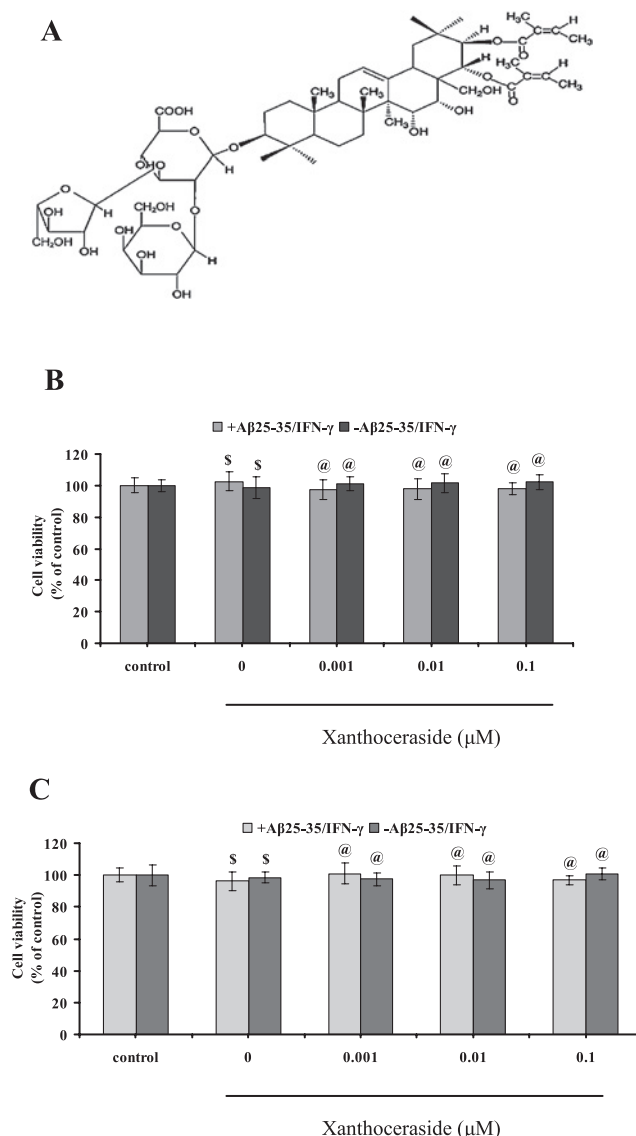


Fig. 1. Chemical structure of xanthoceraside and viability of microglia by treatment with xanthoceraside and/or $A\beta_{25-35}$ (10 μ M) / IFN- γ (10 U·mL⁻¹) using the MTT assay. A: Chemical structure of xanthoceraside. B and C: Primary microglia (B) and N9 microglia (C) were pretreated with xanthoceraside (0.001, 0.01, 0.1 μ M) for 16 h before incubating with $A\beta_{25-35}$ (10 μ M) / IFN- γ (10 U·mL⁻¹) for 24 h, and then cell viability was measured using the MTT assay. The results are shown as percentages of the control. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ^SNot significantly different from the control group. [@]Not significantly different from the $A\beta_{25-35}$ /IFN- γ -treated group. Significance was determined by Student's *t*-test.

stored at -20°C . The final concentration of DMSO in all of the assays was not greater than 0.1%. DMEM, MEM and fetal bovine serum (FBS) were obtained from GIBCO (Carlsbad, CA, USA). Poly-D-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT), DMSO, and $A\beta_{25-35}$ were purchased from Sigma (St. Louis, MO, USA). IFN- γ was purchased from Peprotech (Rocky Hill, NJ, USA). CD11b antibody was obtained from Serotec (Oxford, UK). Antibodies against iNOS, cyclooxygenase-2 (COX-2), myeloid differentiation protein 88 (MyD88), inhibitor- κ B-kinase (IKK), NF- κ B P65, NF- κ B P50, inhibitor of κ B α (I κ B α), β -actin, ERK, p38, JNK, phosphorylated-ERK, phosphorylated-p38, and phosphorylated-JNK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TRIzol reagent was from Invitrogen Co. (Carlsbad, CA, USA). Revert Aid TM First Strand cDNA Synthesis Kit was from Fermentas (Burlington, Ontario, Canada). Taq polymerase was from Takara Biotechnology (Dalian, China). TNF- α and IL-1 β enzyme-linked immunosorbent assay (ELISA) kits were from Diaclone (Besancon, France). All other chemicals were of analytical grade.

Methods

Hashioka et al. (9) stated in their paper that a great increase (about 10-fold) in TNF- α accumulation is induced in primary cultured rat microglia after exposure to $A\beta_{25-35}$ plus IFN- γ at 37°C for 24 h; therefore the experiments were performed after 24-h incubation with $A\beta_{25-35}$ plus IFN- γ . In our preliminary experiments, we found that $A\beta_{25-35}$ plus IFN- γ induced the increases in IL-1 β , TNF- α , and NO expression in microglia after 24 h without affecting the cell viability of microglia, while pretreatment with xanthoceraside for 16 h suppressed the release of IL-1 β , TNF- α , and NO in a concentration-dependent manner. Therefore, all of the experiments in our studies were performed after 24-h incubation with $A\beta_{25-35}$ plus IFN- γ , which was followed by 16-h pretreatment with xanthoceraside.

Cell culture

The murine microglial cell line N9 (Shanghai Bioleaf Biotech Co., Ltd.) was grown and maintained in 95% air and 5% CO₂ atmosphere in DMEM containing 10% FBS and 100 U/mL of antibiotics (streptomycin and penicillin) at 37°C.

Primary mixed glial cells were isolated from the brain of 1- to 2-day Sprague-Dawley rats according to Chen et al. (16). The procedures followed were in accordance with institutional guidelines. Briefly, after sterilization with 75% ethanol for 3 min, the brain was dissected, and the meninges were carefully removed. Cerebral cortices were triturated slowly in serum-free MEM using a pipette. The cells were cultured for 8 or 9 day in 75 cm² flasks coated with poly-D-lysine (1 μ g/mL) and then maintained in MEM supplemented with 10% FBS and 100 U/mL (penicillin and streptomycin) at 37°C and

5% CO₂ atmosphere. The flasks were shaken at 200 rpm for 2 min to separate the microglia. The medium was collected in a sterile culture tube and centrifuged at 1400 rpm for 120 s. In a 96-well plate, 3×10^4 cells per well were seeded. After 8 h, the microglia were used for experiments. The purity of the microglia-enriched cultures was over 98%, as confirmed by immunostaining with CD11b antibody.

MTT reduction assay

Cell viability was assessed by the MTT assay. In brief, primary microglia (3×10^4 cells/well) and N9 microglia (5×10^4 cells/well) were seeded in a 96-well plate and pretreated with different concentrations of xanthoceraside (0, 0.001, 0.01, or 0.1 μ M) for 16 h, followed by 24 h of treatment with or without $A\beta_{25-35}$ (10 μ M) / IFN- γ (10 U·mL⁻¹). After various treatments, the medium was removed and the cells were incubated with 5 mg/mL MTT in phosphate-buffered saline (PBS) at 37°C for 4 h. The resulting dark blue crystals were dissolved with an equal volume of DMSO. The optical density of the resulting solutions was determined at a test wavelength of 490 nm using a SPECTRA Reader (TECAN, Grödig, Austria).

Measurement of nitrite levels

For NO (nitrite/nitrate) assay, cells were induced by $A\beta_{25-35}$ (10 μ M) / IFN- γ (10 U·mL⁻¹) in the presence of xanthoceraside (0, 0.001, 0.01, or 0.1 μ M) for 24 h. Supernatants collected from the microglia were evaluated for NO determination using the Griess reagent (Beyotime, Beijing, China) and NO level was determined at 540 nm using a SPECTRA Reader. Nitrite concentrations were calculated with reference to the standard solutions of sodium nitrite prepared in cell-culture medium.

ELISA

The concentrations of IL-1 β and TNF- α in the supernatants were determined with ELISA (R&D Systems, Besancon, France) according to the manufacturer's protocol.

Western blot analysis

N9 microglia were pretreated with xanthoceraside (0, 0.01, and 0.1 μ M) for 16 h and induced by $A\beta_{25-35}$ (10 μ M) / IFN- γ (10 U·mL⁻¹) for 24 h. Cells were washed thrice with PBS and lysed with lysis buffer (Beyotime). Total protein in the cell extracts were determined using the Bradford assay (Beyotime). Protein extracts (50 μ g) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After the incubation at room temperature with 5% nonfat milk in

PBS for 2 h, the membranes were incubated again with the indicated primary antibodies overnight at 4°C for iNOS (1:500), COX-2 (1:500), NF- κ B p65 (1:1000), NF- κ B p50 (1:1000), I κ B α (1:1000), IKK (1:1000), ERK (1:2000), JNK (1:1500), p38 (1:1500), phospho-ERK (1:1000), phospho-JNK (1:1000), phospho-p38 (1:1000), MyD88 (1:1000), and β -actin (1:1000). The membranes were then incubated with the appropriate dilution of horseradish peroxidase-labeled IgG (Santa Cruz) at room temperature for 2 h. Protein bands were developed using electrochemiluminescent western blotting reagents (Cwbiotech, Beijing, China). The intensity was quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA) and corrected with the corresponding β -actin level. The results were expressed as a percentage of the control.

Isolation of total RNA and reverse transcriptase–polymerase chain reaction (RT-PCR)

N9 cells (3×10^5 cells/well) were cultured in six-well plates, and total RNA was extracted with TRIzol. For RT-PCR, 2.0 mg of total RNA was reverse-transcribed using a first-strand cDNA synthesis system. cDNA products were amplified by PCR with specific primers of TLR-2, iNOS, TNF- α , IL-1 β , COX-2, and GAPDH. The primer sequences used are presented in Table 1. After 35 cycles, the amplified PCR products were separated on 2% agarose gels and then visualized with ethidium bromide under UV light. The intensity was quantified using Quantity One 4.6.2 software and corrected with the corresponding GAPDH level. The results were expressed as a percentage of the control.

Immunofluorescence assay

According to the cellular NF- κ B translocation kit (Beyotime) procedure provided by the manufacturer, N9 cells were immunofluorescence-labeled according to the methods of Xu et al. (17) and Kim et al. (18). Briefly, N9 microglia cells (1×10^5 cells/well) were cultured

on sterile cover slips in 24-well plates and treated with xanthoceraside and A β_{25-35} (10 μ M) / IFN- γ (10 U·mL⁻¹). Before being incubated with a blocking buffer at room temperature for 1 h, the cells were washed thrice and fixed 30 min at 60 min after the A β_{25-35} /IFN- γ treatment. The cells were treated with NF- κ B p65 antibody for 1 h, washed thrice, incubated with a Cy3-conjugated secondary antibody for 1 h at room temperature, and then washed thrice. The cells were stained with 4'-6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature and then washed thrice. Finally, the cells were viewed under a fluorescence microscope. The p65 protein and nuclei exhibited red and blue fluorescence excitation wavelengths of 540 nm and 350 nm for Cy3 and DAPI, respectively.

Statistical analyses

Results are expressed as means $\pm$ standard deviation (S.D.) from at least three separate experiments in triplicate. Statistical difference was determined using ANOVA followed by Fisher's least significant difference (LSD) multiple comparisons test; $P < 0.05$ was considered to indicate statistical significance. The data were analyzed with SPSS 17.0.

Results

Xanthoceraside does not affect the cell viability of microglia

The viability of both primary cultured rat microglia and N9 microglia, which were incubated with A β_{25-35} /IFN- γ for 24 h with xanthoceraside (0, 0.001, 0.01, and 0.1 μ M) was measured using the MTT assay to assess the cytotoxic effects of xanthoceraside on microglia. The results showed that compared with the controls, xanthoceraside at the concentration range of 0.001 – 0.1 μ M did not significantly affect the cell viability of microglia (Fig. 1: B and C).

Table 1. PCR preimers used

Gene target	forward sequence	reverse sequence	Size (bp)
TLR-2	5'-GGA GGT TGC ATA TCC CCC AG-3'	5'-AGC AAC ACA GGG AAC AAC GA-3'	562
COX-2	5'-CAG CAA ATC CTT GCT GTT CC-3'	5'-TGG GCA AAG AAT GCA AAC ATC-3'	426
IL-1 β	5'-TGC TGG TGT GTG ACG TTC CC-3'	5'-TGA GGC CCA AGG CCA CAG GTA-3'	201
iNOS	5'-TTT GCC ACG GAC GAG ACG GAT-3'	5'-GCC ACT GAC ACT TCG CAC AAA-3'	352
TNF- α	5'-CCA CAC CGT CAG CCG ATT TG-3'	5'-CAC CCA TTC CCT TCA CAG AGC-3'	234
GAPDH	5'-GCA GTG GCA AAG TGG AGA TGG-3'	5'-TGC AGG ATC CAT TGC TGA CA-3'	357

TLR-2, Toll-like receptor-2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

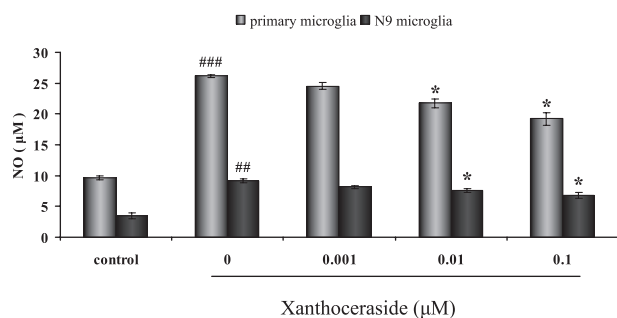


Fig. 2. Effects of xanthoceraside on production of NO induced by $A\beta_{25-35}$ /IFN- γ on primary microglia and N9 microglia. Cells were pretreated with xanthoceraside (0.001, 0.01, 0.1 μM) for 16 h before incubating with $A\beta_{25-35}$ (10 μM) / IFN- γ (10 $\text{U}\cdot\text{mL}^{-1}$) for 24 h, and then the supernatants collected from microglia were measured for NO by using the Griess reagent. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ### P < 0.01 and ### P < 0.001: compared with the control group, * P < 0.05: compared with the $A\beta_{25-35}$ /IFN- γ -treated group by ANOVA followed by the LSD multiple comparisons test.

Xanthoceraside inhibits NO production in microglia

Activated microglia produce high levels of NO, which strongly synergizes with hypoxia to induce neuronal death by causing the inhibition of the mitochondrial cytochrome oxidase in neurons (19). Therefore, inhibiting NO production could be a key mechanism to suppress neuroinflammation relevant to AD. Our results showed that compared with the controls (primary microglia: $9.6 \pm 0.29 \mu\text{M}$, N9 microglia: $3.43 \pm 0.46 \mu\text{M}$), $A\beta_{25-35}$ /IFN- γ treatment markedly induced NO production (primary microglia: $26.18 \pm 0.25 \mu\text{M}$, P < 0.001; N9 microglia: $9.18 \pm 0.33 \mu\text{M}$, P < 0.01) (Fig. 2). Pretreatment with xanthoceraside (0.01 and 0.1 μM) significantly suppressed the increase of NO production in primary microglia (0.01: $21.75 \pm 0.69 \mu\text{M}$, P < 0.05; 0.1: $19.20 \pm 0.98 \mu\text{M}$, P < 0.05) and N9 microglia (0.01: $7.62 \pm 0.29 \mu\text{M}$, P < 0.05; 0.1: $6.79 \pm 0.49 \mu\text{M}$, P < 0.05), respectively. Moreover, xanthoceraside did not affect the cell viability of microglia. Thus, the inhibitory effect of xanthoceraside on NO production was not due to any cytotoxic action on $A\beta_{25-35}$ /IFN- γ -induced microglia.

Xanthoceraside attenuates IL-1 β and TNF- α production in microglia

Most studies convincingly demonstrated that activated microglia release pro-inflammatory cytokines IL-1 β and TNF- α (20). Primary microglia pretreated with xanthoceraside (0, 0.001, 0.01, and 0.1 μM) for 16 h were further incubated with $A\beta_{25-35}$ /IFN- γ for 24 h. The release of pro-inflammatory cytokines into the culture supernatants were evaluated by ELISA. As shown in Fig. 3, A and C, $A\beta_{25-35}$ /IFN- γ induced the increase in IL-1 β

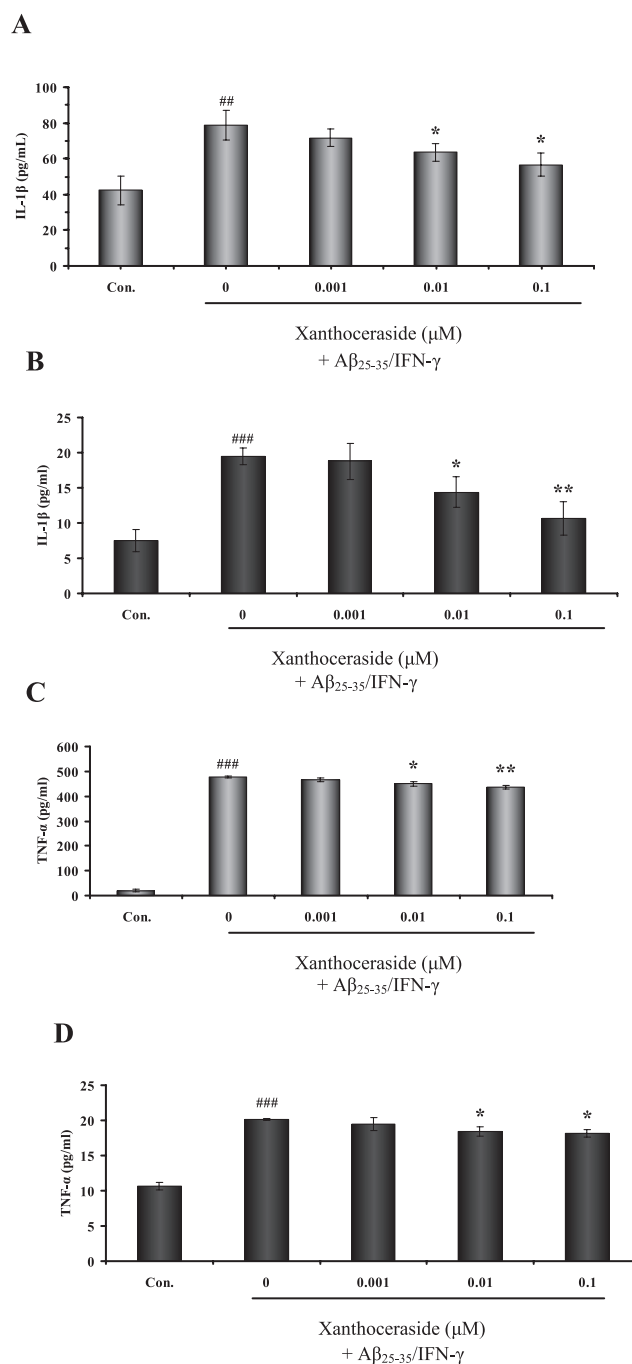


Fig. 3. Effects of xanthoceraside on IL-1 β and TNF- α production. Primary microglia and N9 microglia were pretreated with various concentrations of xanthoceraside (0.001, 0.01, 0.1 μM) for 16 h before incubating with $A\beta_{25-35}$ (10 μM) / IFN- γ (10 $\text{U}\cdot\text{mL}^{-1}$). The cultured supernatants were collected and analyzed using ELISA. The absorbance was determined spectrophotometrically at 450 nm. A and C: IL-1 β and TNF- α production were measured in $A\beta_{25-35}$ /IFN- γ -induced primary microglia. B and D: IL-1 β and TNF- α production were measured in $A\beta_{25-35}$ /IFN- γ -induced N9 microglia. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ### P < 0.01 and ### P < 0.001: compared with the control group, * P < 0.05 and ** P < 0.01: compared with the $A\beta_{25-35}$ /IFN- γ -treated group by ANOVA followed by the LSD multiple comparisons test.

(78.74 ± 8.41 pg/mL) and TNF- α (477 ± 5.27 pg/mL) expression in primary microglia, whereas xanthoceraside suppressed the release of IL-1 β and TNF- α (IL-1 β : 0.01: 63.56 ± 4.84 pg/mL, $P < 0.05$ and 0.1: 56.52 ± 6.51 pg/mL, $P < 0.05$; TNF- α : 0.01: 449.75 ± 8.93 pg/mL, $P < 0.05$ and 0.1: 434.83 ± 8.05 pg/mL, $P < 0.01$) in a concentration-dependent manner. Furthermore, N9 microglia pretreated with xanthoceraside (0, 0.001, 0.01, and 0.1 μ M) for 16 h were further stimulated with A β_{25-35} /IFN- γ for another 24 h. As shown in Fig. 3, B and D, unstimulated macrophages produced a background level of IL-1 β (7.54 ± 1.55 μ M) and TNF- α (10.61 ± 0.55 μ M) in the culture medium. When N9 cells were stimulated with A β_{25-35} /IFN- γ for 24 h, the levels of IL-1 β and TNF- α were markedly increased in the culture medium to 19.49 ± 1.24 pg/mL ($P < 0.001$) and 20.13 ± 0.18 pg/mL ($P < 0.001$), respectively. Xanthoceraside (0.01 and 0.1 μ M) suppressed the increase in IL-1 β and TNF- α (IL-1 β : 0.01: 14.36 ± 2.19 pg/mL, $P < 0.05$ and 0.1: 10.64 ± 2.35 pg/mL, $P < 0.01$; TNF- α : 0.01: 18.42 ± 0.69 pg/mL, $P < 0.05$ and 0.1: 17.01 ± 0.54 pg/mL, $P < 0.05$) in a concentration-dependent manner. RT-PCR analysis was also performed to assess the effect of xanthoceraside on TNF- α and IL-1 β mRNA expression. In line with the results of these cytokine protein levels, A β_{25-35} /IFN- γ significantly increased the levels of TNF- α and IL-1 β mRNA expression (TNF- α : $237.07\% \pm 12.78\%$ of the control, $P < 0.001$; IL-1 β : $268.42\% \pm 9.65\%$ of the control, $P < 0.001$). Treatment with xanthoceraside (0.01 and 0.1 μ M) significantly induced the down-regulation of TNF- α and IL-1 β mRNA expression in a concentration-dependent manner (TNF- α : 0.01: $200.21\% \pm 11.31\%$ of the control, $P < 0.05$ and 0.1: $171.76\% \pm 13.52\%$ of the control, $P < 0.01$; IL-1 β : 0.01: $195.28\% \pm 15.37\%$ of the control, $P < 0.01$ and 0.1: $163.39\% \pm 19.21\%$ of the control, $P < 0.01$) (Fig. 4). These findings suggested that xanthoceraside possessed a suppression effect against pro-inflammatory cytokine production from activated microglia at the transcriptional level.

Xanthoceraside inhibits iNOS and COX-2 expression in N9 cells

The effect of xanthoceraside on iNOS and COX-2 protein expression were elucidated by western blotting analysis. As shown in Fig. 5A, A β_{25-35} /IFN- γ -stimulated N9 microglia increased iNOS and COX-2 protein expression (iNOS: $287.34\% \pm 18.88\%$ of the control, $P < 0.001$; COX-2: $242.79\% \pm 16.22\%$ of the control, $P < 0.001$). However, these increases were markedly attenuated by the pretreatment of N9 microglia with xanthoceraside at 0.01 and 0.1 μ M (iNOS: 0.01: $205.80\% \pm 21.19\%$ of the control, $P < 0.01$ and 0.1:

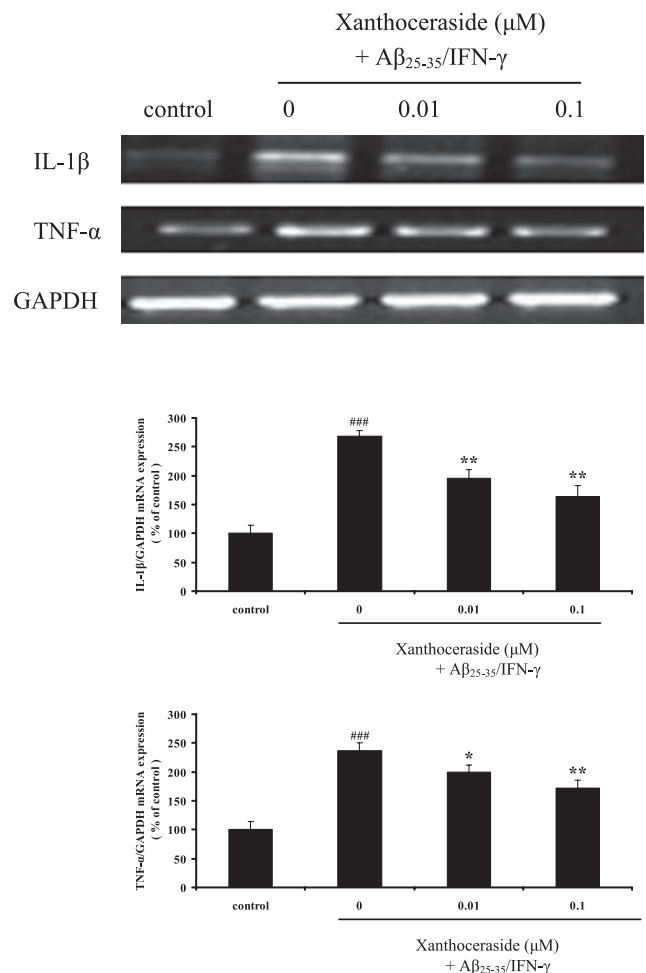


Fig. 4. Effect of xanthoceraside on pro-inflammatory cytokines IL-1 β and TNF- α mRNA expression. N9 microglia were pretreated with xanthoceraside (0.01, 0.1 μ M) for 16 h and then stimulated with A β_{25-35} (10 μ M) / IFN- γ (10 U/mL) for 24 h. Total RNA was prepared for RT-PCR analysis and determination of IL-1 β and TNF- α mRNA expression from A β_{25-35} /IFN- γ -induced N9 microglia. GAPDH was used as an internal control. The intensity was quantified by densitometry and corrected with the corresponding GAPDH level. The results were expressed as a percentage of the control. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ### $P < 0.001$: compared with control group, * $P < 0.05$ and ** $P < 0.01$: compared with A β_{25-35} /IFN- γ -treated group by ANOVA followed by LSD multiple comparisons test.

$167.69\% \pm 30.82\%$ of the control, $P < 0.001$; COX-2: 0.01: $185.58\% \pm 11.72\%$ of the control, $P < 0.001$ and 0.1: $155.15\% \pm 19.96\%$ of the control, $P < 0.001$). In addition, A β_{25-35} /IFN- γ -induced iNOS and COX-2 mRNA overexpression were analyzed by semi-quantitative RT-PCR. As shown in Fig. 5B, A β_{25-35} /IFN- γ -induced N9 cells significantly increased the levels of iNOS mRNA expression ($221.3\% \pm 18.81\%$ of the control, $P < 0.001$) and COX-2 mRNA expression ($331.35\% \pm 23.34\%$ of the control, $P < 0.001$). Pretreatment with xanthoceraside

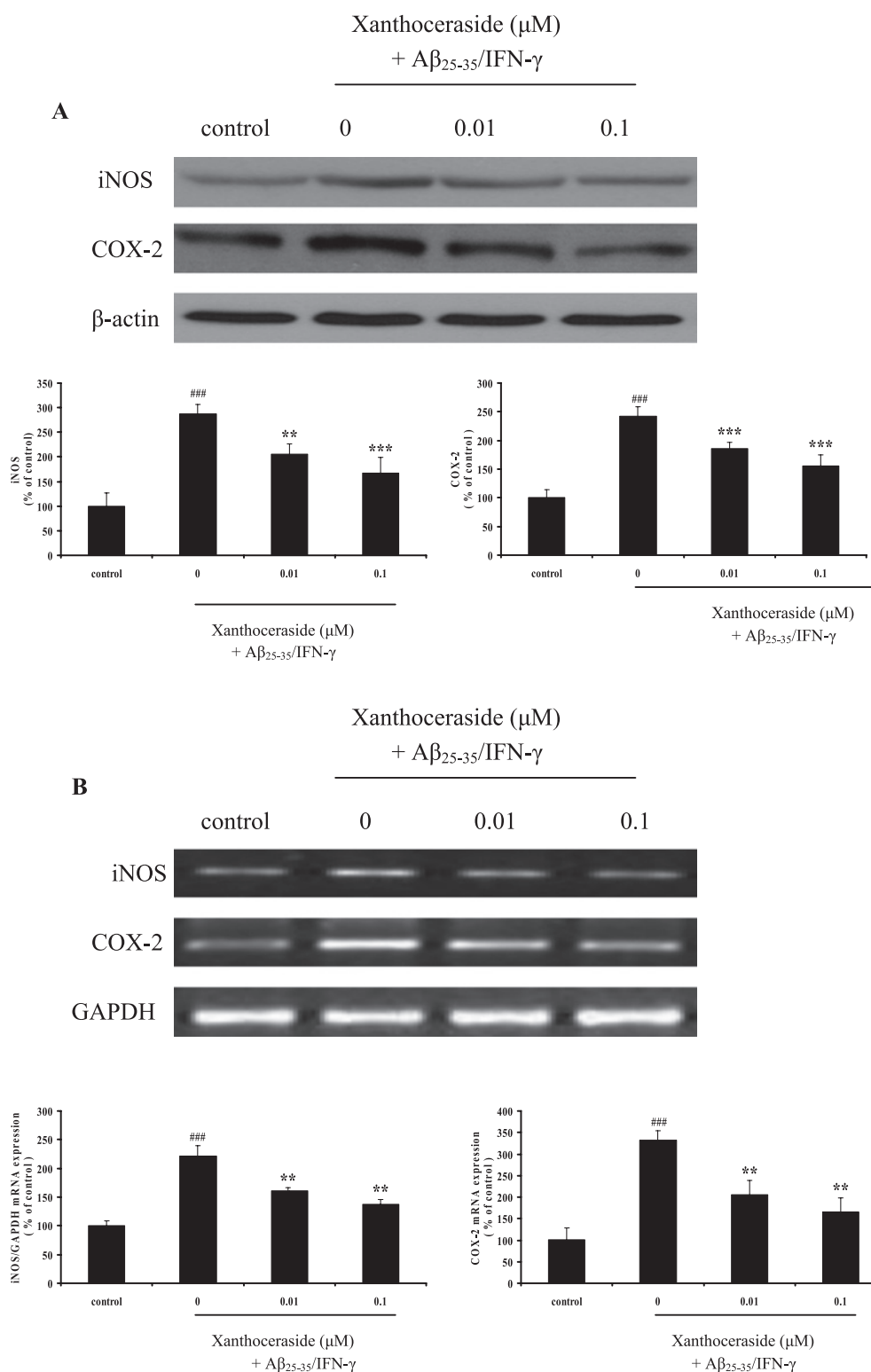


Fig. 5. Effects of xanthoceraside on $A\beta_{25-35}/\text{IFN-}\gamma$ -induced COX-2 and iNOS protein (A) and mRNA (B) expression in N9 microglia. A: The cells were pretreated with xanthoceraside (0.01, 0.1 μM) for 16 h and then stimulated with $A\beta_{25-35}$ (10 μM) / $\text{IFN-}\gamma$ (10 $\text{U}\cdot\text{mL}^{-1}$) for 24 h. The whole cell extracts were prepared for western blot analysis for the indicated proteins. β -Actin was used as an internal control. The intensity was quantified by densitometry and corrected with the corresponding β -actin level. The results were expressed as a percentage of the control. B: Total RNA was isolated and the mRNA transcript levels were measured by RT-PCR. GAPDH was used as an internal control. The results were expressed as a percentage of the control. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ^{###} $P < 0.001$: compared with the control group, ^{**} $P < 0.01$ and ^{***} $P < 0.001$: compared with the $A\beta_{25-35}/\text{IFN-}\gamma$ -treated group by ANOVA followed by the LSD multiple comparisons test.

at 0.01 and 0.1 μM significantly decreased the $A\beta_{25-35}/\text{IFN-}\gamma$ -induced iNOS and COX-2 mRNA expression (iNOS: 0.01: $160.86\% \pm 6.08\%$ of the control, $P < 0.01$ and $137.82\% \pm 8.58\%$ of the control, $P < 0.01$, respec-

tively; COX-2: 0.01: $205.39\% \pm 32.36\%$ of the control, $P < 0.01$ and 0.1: $164.98\% \pm 32.91\%$ of the control, $P < 0.01$, respectively), in a dose-dependent manner.

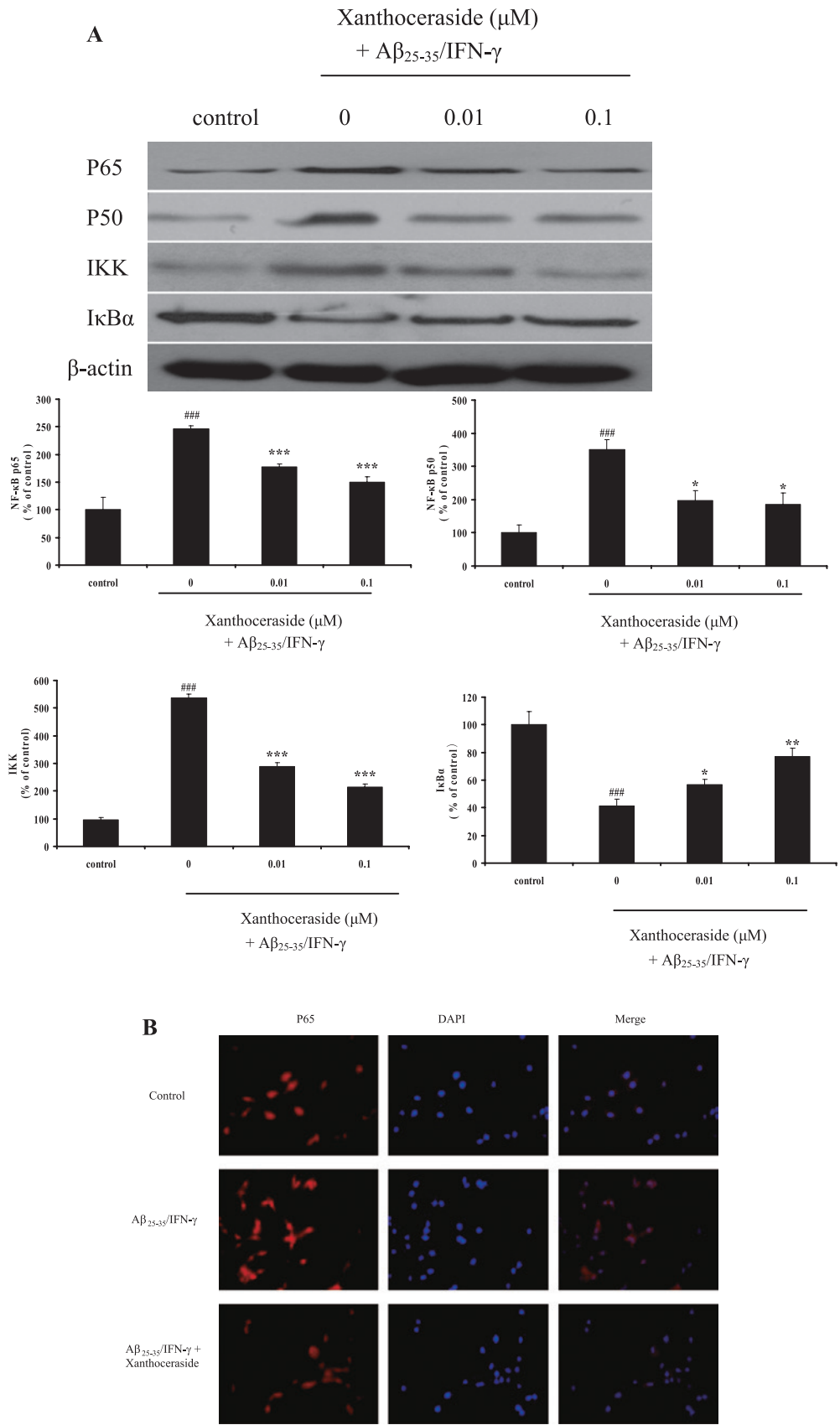
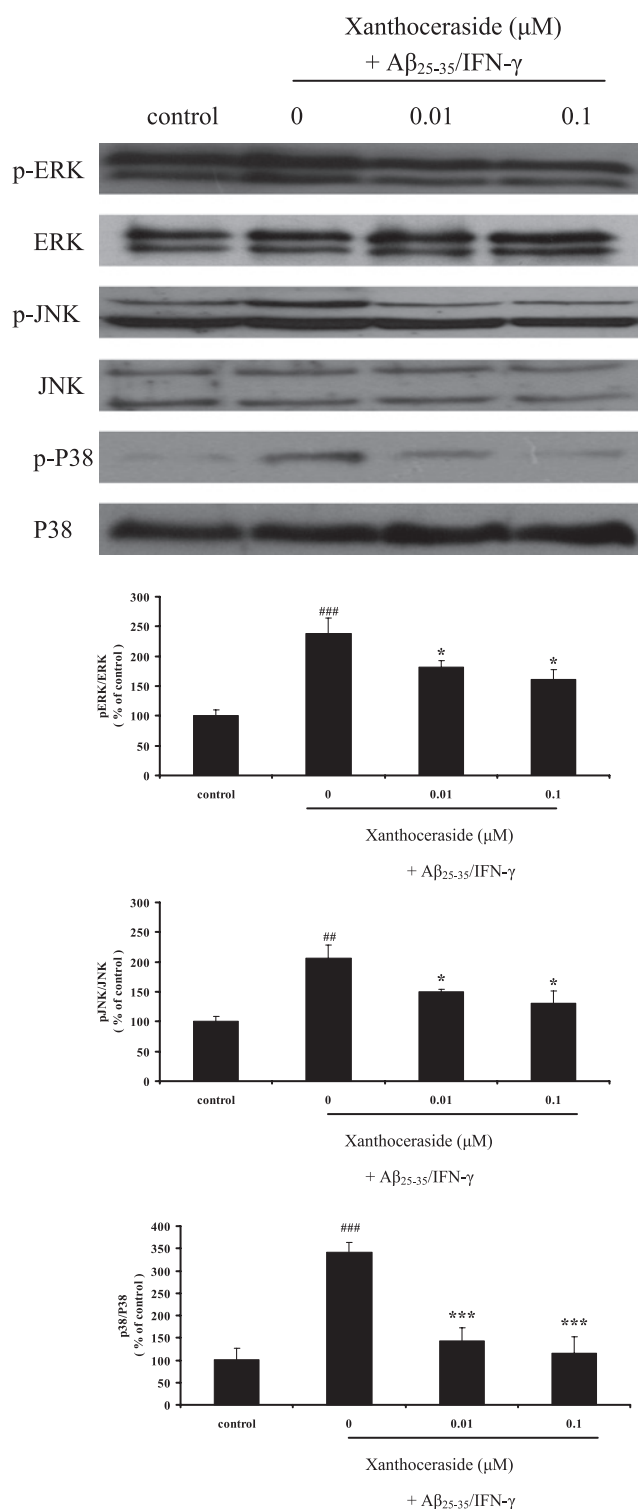


Fig. 6. Effect of xanthoceraside on NF- κB activation in $\text{A}\beta_{25-35}/\text{IFN-}\gamma$ -stimulated N9 microglia. A: Western blot analysis of NF- κB p50 and p65 subunits, IKK and I $\kappa\text{B}\alpha$ in the whole cell extracts of N9 microglia treated with xanthoceraside (0.01, 0.1 μM) for 16 h followed by the stimulation with $\text{A}\beta_{25-35}$ (10 μM) / IFN- γ (10 U $\cdot\text{mL}^{-1}$) for 24 h. β -Actin was used as an internal control. The intensity was quantified by densitometry and corrected with the corresponding β -actin level. The results were expressed as a percentage of the control. B: Sub-cellular location of NF- κB p65 subunit was determined by immunofluorescence assay. The cells were treated with xanthoceraside (0.1 μM) for 16 h followed by the stimulation with $\text{A}\beta_{25-35}$ (10 μM) / IFN- γ (10 U $\cdot\text{mL}^{-1}$) for 1 h. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ^{###} $P < 0.001$: compared with the control group, ^{*} $P < 0.05$, ^{**} $P < 0.01$, and ^{***} $P < 0.001$: compared with the $\text{A}\beta_{25-35}/\text{IFN-}\gamma$ -treated group by ANOVA followed by the LSD multiple comparisons test.

Xanthoceraside inhibits NF- κ B activation in N9 microglia

Activation of NF- κ B is necessary for the induction of iNOS and COX-2 genes as well as for the transcription of pro-inflammatory molecules. Therefore, we investigated the role of NF- κ B activation in $A\beta_{25-35}$ /IFN- γ -induced microglia by western blotting analysis. As



shown in Fig. 6A, stimulation of N9 microglia with $A\beta_{25-35}$ /IFN- γ increased the protein levels in the nuclear translocations of NF- κ B p50, and p65 subunits. However, xanthoceraside attenuated their protein expressions. The immunofluorescence assay was used to further characterize the effect of xanthoceraside on NF- κ B nuclear translocation. As shown in Fig. 6B, xanthoceraside inhibited the $A\beta_{25-35}$ /IFN- γ -induced translocation of p65 into the nucleus. The effects of xanthoceraside on I κ B α and IKK were also evaluated by western blot analysis. The results showed that $A\beta_{25-35}$ /IFN- γ markedly increased degradation of I κ B α and decreased IKK level. However, xanthoceraside significantly blocked I κ B degradation and increased IKK level (Fig. 6A). These results indicated that xanthoceraside suppressed the production of NO, iNOS, COX-2, and pro-inflammatory cytokines (TNF- α , IL-1 β) via the inhibition of NF- κ B activation and degradation of I κ B α in $A\beta_{25-35}$ /IFN- γ -stimulated N9 cells.

Xanthoceraside inhibits MAPK activation in N9 microglia

Three major MAPK molecules, ERK, JNK, and p38, were detected to further examine whether the inhibitory mechanism of xanthoceraside against microglial activation occurs via alteration of MAPK activity. The levels of phosphorylated-ERK, -JNK, and -p38 were significantly increased in N9 microglia with $A\beta_{25-35}$ /IFN- γ . Xanthoceraside pretreatment significantly attenuated the $A\beta_{25-35}$ /IFN- γ -induced phosphorylation of ERK, JNK, and p38 (Fig. 7), whereas their non-phosphorylated forms were not changed. The results suggested that xanthoceraside-mediated attenuation of pro-inflammatory mediators was associated with MAPK signal transduction pathways in $A\beta_{25-35}$ /IFN- γ -induced N9 microglial cells.

Xanthoceraside inhibits TLR2 overexpression by the MyD88-dependent pathway

Toll-like receptors (TLRs) are important in the recognition of pathogens by initiating responses to host-derived, endogenous ligands (21, 22). We evaluated the

Fig. 7. Effect of xanthoceraside on the phosphorylation of MAPK in $A\beta_{25-35}$ /IFN- γ -stimulated N9 microglia. Cells were pretreated with xanthoceraside (0.01, 0.1 μ M) for 16 h and then stimulated with $A\beta_{25-35}$ (10 μ M) / IFN- γ (10 U·mL⁻¹) for 24 h. The MAPK and phosphorylation of MAPK were detected by western blotting analysis with antibodies against p-ERK, ERK, p-JNK, JNK, p-P38, and P38. β -Actin was used as an internal control. The intensity was quantified by densitometry and corrected with the corresponding β -actin level. The results were expressed as a percentage of the control. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ^{##} $P < 0.01$ and ^{###} $P < 0.001$: compared with the control group, ^{*} $P < 0.05$ and ^{***} $P < 0.001$: compared with the $A\beta_{25-35}$ /IFN- γ -treated group by ANOVA followed by the LSD multiple comparisons test.

effect of xanthoceraside on TLR2 expression in N9 microglia using RT-PCR. $A\beta_{25-35}/\text{IFN-}\gamma$ significantly increased TLR2 mRNA expression, whereas xanthocera-

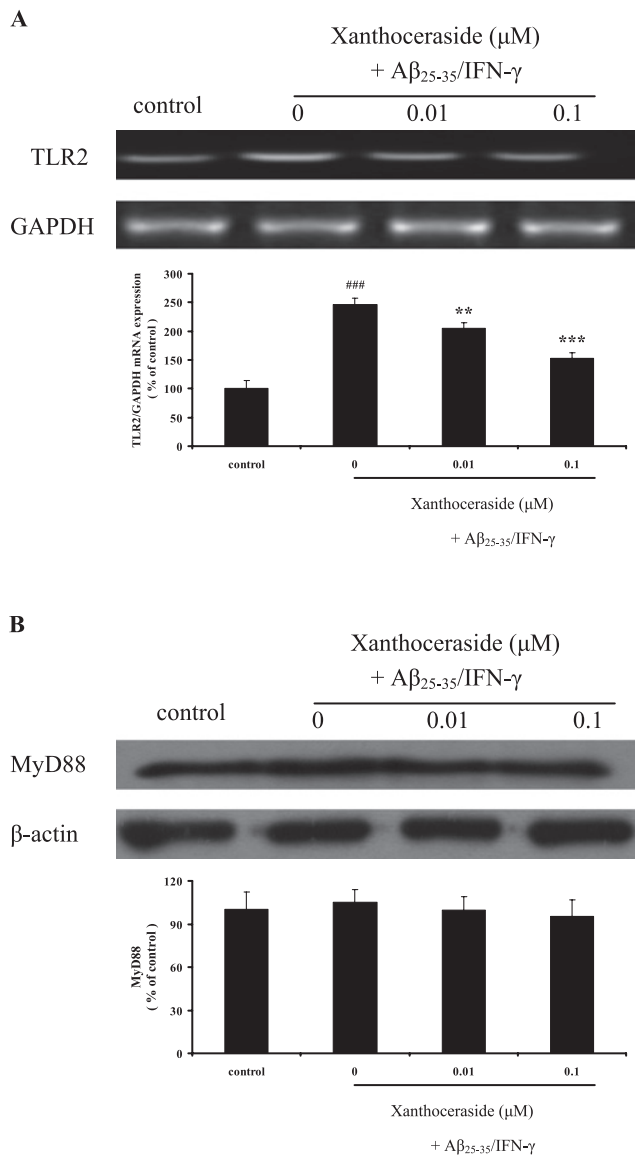


Fig. 8. Effect of xanthoceraside on $A\beta_{25-35}/\text{IFN-}\gamma$ -induced expression of TLR2/MyD88. The cells were pretreated with xanthoceraside (0.01, 0.1 μM) for 16 h and then stimulated with $A\beta_{25-35}$ (10 μM) / $\text{IFN-}\gamma$ (10 $\text{U}\cdot\text{mL}^{-1}$) for 24 h. A: Total RNA was isolated and the mRNA transcript levels were measured by RT-PCR for TLR2. GAPDH was used as an internal control. The results were expressed as a percentage of the control. B: The whole cell extracts were prepared for western blotting analysis for MyD88 protein. β -Actin was used as an internal control. The intensity was quantified by densitometry and corrected with the corresponding β -actin level. The results were expressed as a percentage of the control. Values are given as the mean $\pm$ S.D. from 3 independent experiments. ^{###} $P < 0.001$: compared with control group, ^{**} $P < 0.01$ and ^{***} $P < 0.001$: compared with the $A\beta_{25-35}/\text{IFN-}\gamma$ -treated group by ANOVA followed by the LSD multiple comparisons test.

side pretreatment decreased TLR2 gene expression in a concentration-dependent manner (Fig. 8A). Furthermore, xanthoceraside did not affect the expression of MyD88, the immediate adaptor molecule to TLR2, in $A\beta_{25-35}/\text{IFN-}\gamma$ -induced N9 microglia (Fig. 8B). The results suggested that the inhibitory effect of xanthoceraside on pro-inflammatory mediators was possibly mediated through TLR2 receptor/MyD88 in $A\beta_{25-35}/\text{IFN-}\gamma$ -stimulated N9 microglial cells.

Discussion

Although in vitro study has some disadvantages, it permits a more detailed or more convenient analysis than can be done with whole organisms and is still widely used in the world by numerous researchers. Our previous study demonstrated that xanthoceraside could alleviate the $A\beta_{25-35}$ -induced impairment of learning and memory, oxidative stress, and inflammation in mice (14, 15). In order to further explore the mechanism that underlines its anti-oxidative and anti-inflammatory effect, we used both primary and cultured microglia cells in this study.

Evidences have found that microglia are activated around the amyloid plaques in AD brain (1), and these activated microglial cells secrete several pro-inflammatory cytokines and neurotoxic mediators associated with the occurrence and exacerbation of AD (23). A number of infiltrating T cells have been shown to increase in the brain of AD. The immune cells of AD patients have the potency for overproduction of $\text{IFN-}\gamma$ (9), and $\text{IFN-}\gamma$ elicited pro-inflammatory cytokine secretion in microglia (24). According to such evidence, our experimental method using $\text{IFN-}\gamma$ thus seems to be consistent with the pathophysiologic microenvironment in the AD brain. A previous study has also shown that a variety of $A\beta$ peptides, in the presence of $\text{IFN-}\gamma$, can activate microglia with production of pro-inflammatory molecules potentially involved in AD (9). In our preliminary experiment, we found that only $A\beta$ itself couldn't activate microglia, which was consistent with a previous report (25). However, the combination of $\text{IFN-}\gamma$ with $A\beta$ could activate microglia, subsequently leading to the generation of reactive nitrogen intermediates and some pro-inflammatory cytokines such as $\text{TNF-}\alpha$ and $\text{IL-}\beta$. Thus, in the present study, we investigated the effects and mechanisms of xanthoceraside on the activated microglia stimulated by $A\beta_{25-35}/\text{IFN-}\gamma$.

XENP345, a TNF -selective inhibitor, reduced the accumulation of intraneuronal β -amyloid precursor protein (APP) in the affected brain regions of LPS-treated 3xTg AD mice (26). $\text{IL-1}\beta$ not only participates in the inflammatory process of AD, but also decreases long-term potentiation (27) and enhances iNOS expres-

sion (28) in the hippocampus which could impair memory. In the present study, we found that xanthoceraside inhibited both the protein and gene expression of TNF- α and IL-1 β . These findings indicated that xanthoceraside might possess potential anti-inflammatory activities.

Stimulation of iNOS catalyzes the oxidation of L-arginine to L-citrulline and NO, which is required for peroxynitrite production. Peroxynitrite has a critical role in A β -induced neurotoxicity and AD. COXs exist in two isoforms, namely, COX-1 and COX-2, which are encoded by different genes. COX-1 is constitutively expressed in most tissues, while COX-2 is mainly induced in response to infection, injury, or other stresses, and increased during inflammatory processes in AD brains (29). In addition, NO activates COX-2, which enhances the release of pro-inflammatory cytokines (30). Previously, our group demonstrated that xanthoceraside prevents peroxynitrite-mediated damage in A β_{25-35} -induced mice (15). In the present study, xanthoceraside significantly attenuated the protein and mRNA expression of iNOS and COX-2 in a concentration-dependent manner in A β_{25-35} /IFN- γ -induced N9 microglia. These findings suggested that xanthoceraside possibly reduced COX-2 production and peroxynitrite-mediated damage by inhibiting the expression of iNOS.

NF- κ B plays a critical role in the onset of inflammatory responses by regulating the expression of pro-inflammatory genes (31). Under the unstimulated condition, NF- κ B is present in the cytosol and is bound to the I κ B proteins. However, once it is activated, I κ B proteins are phosphorylated by IKK and then are degraded. NF- κ B is translocated to the nucleus, and its target genes are activated. Activated genes regulate the expression of cytokines, including iNOS, COX-2, TNF- α , and IL-1 β (32, 33). In the present study, xanthoceraside inhibited the expression of NF- κ B subunits (p50 and p65). Immunofluorescence assay confirmed the down-regulated protein expression of p65 in the nucleus after xanthoceraside pretreatment. Furthermore, xanthoceraside decreased IKK expression and inhibited I κ B degradation in a concentration-dependent manner. All of these data suggested the anti-inflammatory effects of xanthoceraside and its potential mechanism.

MAPK have an important role in the regulation of cell growth and differentiation as well as in the control of cellular responses to cytokines and stresses, including ERK, JNK, and P38. Moreover, MAPK are involved in the release of immune-related cytotoxic factors, such as iNOS, COX-2, and pro-inflammatory cytokines (34), by controlling the activation of NF- κ B (35). In the current study, we showed that the A β_{25-35} /IFN- γ -induced up-regulated phosphorylation of MAPK were signifi-

cantly attenuated by xanthoceraside pretreatment, which suggested that xanthoceraside might inhibit the release of cytotoxic factors through the attenuation of NF- κ B signaling by the MAPK pathway.

A number of studies support an important role of reactive oxygen species (ROS) in the pathophysiology of AD and demonstrate beneficial effects of antioxidants on the progression of this disease. Intracellular ROS could activate diverse downstream signaling molecules, such as protein kinase C, MAPK, and NF- κ B, to regulate the expression of genes encoding various pro-inflammatory factors (36, 37); and the inflammatory effects might result in the accumulation of intracellular ROS (38). IFN- γ could up-regulate ROS production in microglia via transcribed subunits of the nicotinamide adenine dinucleotide phosphate oxidase complex (39). Our previous study proved that xanthoceraside increased the activities of superoxide dismutase and glutathione peroxidase, thereby ameliorating the accumulation of malonaldehyde in the hippocampus induced by the intracerebroventricular injection of A β_{25-35} (14). These findings suggested that xanthoceraside suppressed A β_{25-35} /IFN- γ -induced pro-inflammatory cytokines expression by improving the activity of the antioxidative system and decreasing ROS production in A β_{25-35} /IFN- γ -induced N9 microglia.

Microglia TLRs have important functions in activating innate immunity and regulating the transition from innate to acquired immune responses (40). An increasing amount of data showed the involvement of TLRs not only in inflammation but also in the modulation of learning, memory, and mood on the pathogenesis of AD (41). TLR2 as a member of the TLR family participates in the A β -induced signaling that occurs during inflammation (42). IFN- γ could augment TLR-induced production of cytokines (43). TLR2-deficient mice showed the decreased pro-inflammatory gene expression (44). Pro-inflammatory cytokines up-regulate TLR2 expression, thereby promoting inflammatory responses. Microglia lacking TLR2 showed no induction of NF- κ B and MAPK following A β (45). Thus, the inhibition of TLR2 expression is a key mechanism in controlling inflammation. MyD88, an adaptor molecule for all TLRs (except for TLR3), serves to bridge to the downstream signaling elements (MAPK or NF- κ B) and induces the expression of pro-inflammatory cytokines (46). In A β -stimulated primary microglia isolated from the brain of TLR2^{-/-} mice, MyD88 expression did not increase, and antisense oligonucleotide MyD88 markedly suppressed MyD88 production and attenuated the mRNA expression of iNOS and TNF- α (47). In the present study, xanthoceraside inhibited TLR2 overexpression but did not affect MyD88 expression in A β_{25-35} /IFN- γ -induced N9 microglia. These results suggested that xanthocera-

side suppressed the production of pro-inflammatory cytokines MyD88-dependently through the TLR2 signal transduction pathway in $A\beta_{25-35}$ /IFN- γ -stimulated microglia.

In summary, our results showed that xanthoceraside inhibited the $A\beta_{25-35}$ /IFN- γ -induced pro-inflammatory cytokine in microglia. The inhibition of $A\beta_{25-35}$ /IFN- γ -induced NO, IL-1 β , and TNF- α production by xanthoceraside in microglia could be attributed to the down-regulation of MAPK and NF- κ B activities through the TLR2/MyD88 pathway. Thus, we concluded that xanthoceraside may possess anti-inflammatory activity that may be a potential therapeutic target for AD treatment.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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