Toll-like receptor 4 signaling is involved in PACAP-induced neuroprotection in BV2 microglial cells under OGD/reoxygenation

Xia Qin*[1,2], Zhen-Quan Sun*[2], Xiao-Jing Dai[2], Shan-Shan Mao[2], Jiao-Li Zhang[2], Meng-Xing Jia[2,3], Yong-Mei Zhang[1,2]

[1]Department of Anesthesia Physiology and [2]Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical College, China, [3]Department of Anesthesiology, Xuzhou Medical College Affiliated Hospital, China

Object: The neuroprotective effects of pituitary adenylate cyclise-activating polypeptide (PACAP) have been well documented *in vivo* and *in vitro*. However, the mechanisms by which PACAP protected microglia from ischemic/hypoxic injury via inhibition of microglia activation remain unclear. Toll-like receptor 4 (TLR4) plays a considerable role in the induction of innate immune and inflammatory responses. The purpose of this study is to investigate the effect of PACAP on the oxygen and glucose deprivation (OGD)/reoxygenation BV2 microglia and to explore the role of TLR4/myeloid differentiation protein 88 (MyD88)/nuclear factor-kappa B (NF-kappaB) pathway in the neuroprotective effects of PACAP.

Methods: We conducted OGD/reoxygenation by placing BV2 microglia into an airtight chamber and in glucose-free medium. BV2 microglia cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay. Western blot was utilized to detect TLR4, MyD88 expression, inhibitory protein of NF-kappaB (IkappaB) phosphorylation/degradation, NF-kappaB activation. Level of tumor necrosis factor-alpha (TNF-alpha) in culture medium was measured with enzyme-linked immunosorbent assay (ELISA). Apoptosis was determined by flow cytometry.

Results: We found that pretreatment with PACAP to BV2 cells immediately before OGD/reoxygenation significantly alleviated microglia hypoxic injury. PACAP inhibited upregulation of TLR4, MyD88 and NF-kappaB in BV2 microglial cells exposed to OGD/reoxygenation. PACAP administration also significantly reduced the production of proinflammatory cytokines and apoptosis in BV2 microglia exposed to OGD/reoxygenation.

Discussion: Pretreatment with PACAP inhibited activation of the TLR4/MyD88/NF-kappaB signaling pathway and decreased inflammatory cytokine levels, as well as apoptosis in microglia, thereby attenuating microglia hypoxic injury. Our results suggested that TLR4-mediated MyD88-dependent signaling pathway contributed to neuroprotection of PACAP to microglia against OGD/reoxygenation.

Keywords: BV2 microglia, OGD/reoxygenation, PACAP, TLR4

Introduction

Hypoxia is one of the important physiological stimuli that are often associated with a variety of pathological states, such as ischemia, respiratory diseases, and tumorigenesis. The developing brain is susceptible to hypoxic damage because of its high oxygen and energy requirements. Hypoxia results in direct brain injury which occurs in the acute hypoxic phase and indirect brain injury occurring after reoxygenation which is mainly attributed to neuro-immunological activation in the central nervous system (CNS).[1]

Microglial cells which are a major glial cell element of the CNS, play a critical role as resident immunocompetent and phagocytic cells and serve as scavenger cells in the event of infection, inflammation, trauma, ischemia, and neurodegeneration in the CNS.[2] Hypoxia that is accompanied by cerebral ischemia not only causes neuronal cell injury, but may also induce pathological microglia activation in the CNS. Thus, it is important to exploit new neuroprotective agents such as those targeting on inhibiting microglia activation after hypoxia.

Pituitary adenylate cyclise-activating polypeptide (PACAP) is a member of the VIP/secretin/glucagon peptide family,[3,4] and exists in forms of 38 and 27 amino-acid residues, with PACAP38 being predominant in human tissues. PACAP is widely distributed

*These authors contributed equally to this work.

Correspondence to: Yong-Mei Zhang, Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou 221002, Jiangsu Province, China. Email: zhangym700@163.com

DOI 10.1179/1743132812Y.0000000028

in the brain and peripheral organs, notably in the endocrine pancreas, gonads, and respiratory and urogenital tracts.[4] Recent evidences have documented the neuroprotective effects of PACAP *in vivo*, as well as *in vitro*. PACAP has been reported to be neuroprotective against global cerebral ischemia,[5,6] focal brain ischemia,[7,8] traumatic brain injuries, and those of neurodegenerative diseases.[9] *In vitro*, PACAP protected cerebellar granule cells against programmed cell death physiologically occurring during cerebellar development, as well as cell death induced by different neurotoxins.[10] PACAP prevented the ethanol-induced apoptotic cell death in rat cerebellar granule neurons.[11] In addition, PACAP could play a protective role to cerebellar granule cells against ceramides and apoptosis induced by oxidative stress.[12] Suk[13] demonstrated that PACAP attenuated inflammatory activation of microglia under hypoxic condition. But the mechanisms by which PACAP protected microglia from ischemic/hypoxic injury via inhibition of microglia activation remain unclear.

Recent evidences have suggested that innate immune and inflammatory responses play a critical role in the pathophysiology of hypoxia in cells.[14–16] Then some studies have demonstrated that Toll-like receptors (TLRs) play a critical role in the induction of innate immune and inflammatory responses.[17–19] TLR4 was consistently expressed in microglia.[20] TLR4 activation leads to the sequential recruitment/activation of the myeloid differentiation protein [(myeloid differentiation protein 88 (MyD88)], the IL-1 receptor-associated kinase, the tumor necrosis factor (TNF) receptor-associated factor 6, and the transforming growth factor-beta-activated kinase 1. The signal may converge on the nuclear factor (NF)-kappaB-inducing kinase, which then activates the IkappaB kinase (IKK) complex. The activated IKK complex phosphorylates IkappaB-alpha at serine residues 32 and 36, which triggers its ubiquination/degradation and subsequent release of NF-kappaB, which then translocates to the nucleus and activates the transcription of kappaB-dependent genes, such as IL-1, IL-6, and TNF-alpha.[21–25] Since innate immune and inflammatory responses contribute to hypoxic injury, it is possible that disruption of TLR4 signal pathway could attenuate microglial hypoxic injury through reducing inflammatory cytokine production. Therefore, we intended to know whether PACAP could inhibit the TLR4/MyD88/NF-kappaB signaling pathway in microglia to decrease inflammatory mediators, thus attenuating the microglia hypoxic injury.

In the present study, we have conducted an *in vitro* oxygen and glucose deprivation/reoxygenation (OGD/R) model to examine whether inhibition of TLR4 signal pathway by PACAP will attenuate microglia hypoxic injury.

Materials and Methods

Materials

PACAP 38 peptide (A1439) was obtained from Sigma Aldrich (St Louis, MO, USA). Goat polyclonal anti-TLR4 (sc-16240), rabbit polyclonal anti-MyD88 (sc-11356), mouse monoclonal anti-NF-kappaB p65 (sc-8008), rabbit polyclonal anti-IkappaB-alpha (sc-371), mouse monoclonal anti-p-IkappaB-alpha (sc-8404), rabbit polyclonal anti-Bcl-2 (sc-492), and rabbit polyclonal anti-Bax (sc-526) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-beta actin monoclonal antibody (TA-09), alkaline phosphatase goat anti-rabbit IgG (ZB-2308), alkaline phosphatase horse anti-mouse IgG (ZB-2310), and alkaline phosphatase rabbit anti-goat IgG (ZB-2311) were purchased from ZSGB-BIO (Beijing, China). PageRulerTM Plus Prestained Protein Ladder (no. SM1811) was purchased from Fermentas. Primary antibody dilution buffer (P0023A), secondary antibody dilution buffer (P0023D), phenylmethanesulfonyl fluoride (ST506), bicinchoninic acid protein assay kit (P0012), SDS–polyacrylamide gel electrophoresis (PAGE) sample loading buffer (P0015), and BCIP/NBT alkaline phosphatase color development kit (C3206) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). TNF-alpha enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium was obtained from GIBCO (Carlsbad, CA, USA). BV2 microglial cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis kit was purchased from Baosai Company (Beijing, China). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was obtained from Amresco (Solon, OH, USA).

Cell culture

The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (volume/volume) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. They were maintained at 37°C in 5% CO_2/air environment. The BV2 microglial cells cultured above were described as control cells and the ones treated by PACAP as PACAP + control cells.

OGD followed by reoxygenation and PACAP administration

For exposure to OGD, BV2 cells were washed with glucose-free phosphate-buffered saline (PBS) twice, then placed in glucose-free medium. BV2 cells were then placed into an airtight chamber, bubbled with an anerobic gas mixture (94% N_2, 5%CO_2, and 1% O_2; CO_2 Incubator, Heraeus, Germany) at 37°C. After 3 hours of OGD exposure, BV2 cells were reoxygenated

by high glucose DMEM and incubation under normoxic conditions for 12 hours. PACAP was added into the medium immediately before OGD/R.

Assessment of myocardial cell viability

BV2 cell viability in response to OGD/R and PACAP treatment was assessed by trypan blue exclusion (TBE) and MTT assays, respectively. The TBE assay assesses the integrity of the cell membrane based on the principle that live cells have intact cell membranes to exclude trypan blue dye, whereas dead cells do not and thus stain blue. For this method, BV2 cells were plated, washed with PBS, harvested by trypsinization and then stained with 0.4% trypan blue for 3 minutes. Cells were then and counted using a hemacytometer under a light microscope. Cellular MTT assay was performed by addition of 25 μl MTT stock solution (5 mg/ml) to each well. After incubation for 4 hours, the MTT solution was carefully decanted off and formazan was dissolved with 200 μl DMSO. The absorbance was measured at 492 nm using a 96-well plate ELISA reader (CliniBio 128; ASYS Hitech, Eugendorf, Austria).

Extraction of nuclear proteins

Cells were collected and resuspended at 4°C in buffer A (0.5M HEPES, 0.5M EDTA, 1M DTT, 10 mM aprotinin, 1M NaF, 1M KCl, 0.5M EGTA, 100 mM PMSF, 10 mM Leupeptin, and 0.1M Na_3VO_4), allowed to swell on ice for 15 minutes, and then vortexed for 1 minutes. Samples were centrifuged and the supernatant containing cytosolic proteins was removed. The pellet was resuspended in cold buffer C (0.5M HEPES, 0.5M EDTA, 1M DTT, 10 mM aprotinin, 1M NaF, 5M NaCl, 0.5M EGTA, 100 mM PMSF, 10 mM leupeptin, and 0.1M Na_3VO_4) and incubated on ice for 50 minutes for high salt extraction. Cellular debris was removed by centrifugation for 10 minutes at 4°C, and the supernatant containing nuclear proteins was collected. Nuclear extracts were aliquoted and stored at −80°C for western blotting analysis of p65 protein activity.

Western blot analysis

Protein concentrations were determined by the bicinchoninic acid protein assay. Equal amounts of protein were resolved in SDS–PAGE and transferred electrophoretically onto a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% skimmed milk for 2 hours at room temperature, incubated overnight at 4°C with primary antibodies directed against TLR4, MyD88, NF-kappaB p65, IkappaB-alpha, p-IkappaB-alpha (all diluted 1 : 500 in primary antibody dilution buffer) and beta-actin (loading control diluted 1 : 1000 in primary antibody dilution buffer). After the membrane was washed for 5 minutes each for three times in washing buffer, it was incubated in the appropriate AP-conjugated secondary antibody (diluted 1 : 1000 in secondary antibody dilution buffer) for 2 hours at room temperature. The blotted protein bands were visualized by BCIP/NBT Alkaline Phosphatase Color Development Kit (S3771). Developed films were digitized using an Epson Perfection 2480 scanner (Seiko Corp, Nagano, Japan). Optical densities of detected protein were obtained using Image J.

Apoptosis assay

BV2 cells were cultured in a six-well plate at a density of 1×10^4 cells/ml, then the cells were subjected to normoxia or OGD/R culture. Following the experiments, the cells were harvested and washed with cold PBS for 2–3 times. The washed cells were recentrifuged and resuspend in 200 μl binding buffer, and 10 μl V-FITC were added to cell suspension and incubated at room temperature for 15 minutes. After the incubation period, 300 μl binding buffer and 1 μl PI working solution were added into the cells suspension, mixed gently and then the samples were kept on ice. The apoptotic cells (the annexin V-positive and PI-negative cells) were indicated as the percentage of gated cells. As soon as possible, the stained cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA) to measure the fluorescence emission at 530 nm, and then the proportion of apoptotic BV2 cells were read out automatically by the WinMDI 2.9 software.

Cytokine assay

Protein expression of the cytokines in BV2 cell culture supernatants were determined by ELISA kit (Biotechnology Systems). Supernatants from BV2 cells were tested for the secretion of TNF-alpha. ELISAs were performed according to the manufacturer's instructions. The level of sensitivity for cytokine standard curve was less than 13 pg/ml.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using one-way analysis of variance (SPSS 13.0 for Windows). Differences between experimental groups were determined by the Fisher's Student–Newman–Keuls test. Differences between means were considered significant if $P<0.05$.

Results

PACAP increased cell viability in BV2 microglial cells exposed to OGD followed by reoxygenation

We first investigated the effects of different time duration of OGD on BV2 microglial cell viability with MTT assay. Cell viability did not change significantly when PACAP was added into control BV2 microglial cells. We found that MTT OD value decreased significantly in OGD for 6, 12, and 24 hours followed by reoxygenation for 6 hours ($P<0.05$). Cell viability decreased to about 95% after 3 hours of OGD exposure, about 55% after 12 hours,

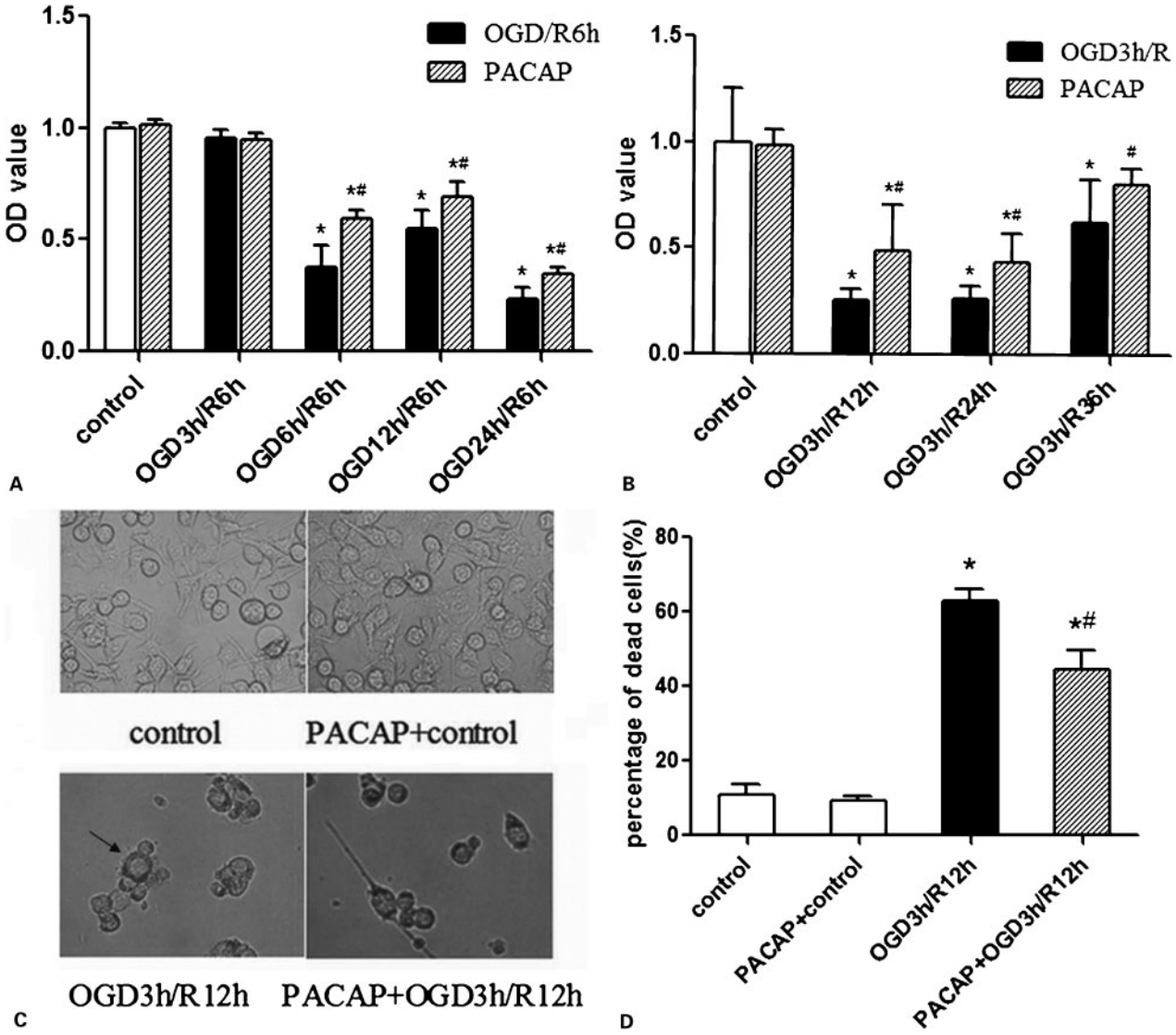

Figure 1 Effect of PACAP on cell viability of BV2 microglia exposed to OGD/R for different OGD and oxygenation time duration. Cells were treated with the indicated concentration of PACAP (100 nM) immediately before OGD/R for different OGD time duration. (A) Effects of different time duration of OGD on BV2 microglial cells. After exposure to OGD for 3, 6, 12, and 24 hours, BV2 cells were reoxygenated by normal DMEM and incubation under normoxic conditions for 6 hours. (B) Effects of different time duration of reoxygenation on BV2 microglial cells. After exposure to OGD for 3 hours, BV2 cells were reoxygenated for 12, 24, and 36 hours. Cell viability was assessed using the MTT assay, and the results are expressed as MTT OD values. Each value indicates the mean ± SD and is representative of results obtained from four independent experiments. *$P<0.05$, as compared with control group. $^{\#}P<0.05$, as compared with OGD/R group. (C) BV2 microglial cells were exposed to OGD for 3 hours followed by reoxygenation for 12 hours. Morphological changes were observed under an inverted microscope (× 400). (D) Exclusion of trypan blue in cells. BV2 cells were incubated with 100 nM PACAP followed by OGD for 3 hours and reoxygenation for 12 hours. The cytosol stained by trypan blue represents the loss of plasma-membrane integrity and cell death. The positive-staining cells were counted under an optical microscope. Results are means ± SD ($n=4$; *$P<0.05$, as compared with control group. $^{\#}P<0.05$, as compared with OGD/R group).

and about 24% after 24 hours OGD. PACAP significantly increased cell viability in BV2 cells under OGD/R at 6-, 12-, and 24-hour OGD timepoints ($P<0.05$), but no dramatic change occurred at 3-hour timepoint (Fig. 1A). Subsequently, we detected the effects of different time duration of reoxygenation on BV2 cell viability with MTT assay. OGD for 3 hours/reoxygenation for 12, 24, and 36 hours all reduced cell viability. PACAP could inhibit the cell viability reduction in OGD for 3 hours followed by reoxygenation for 12, 24, and 36 hours, and augment cell viability by 91, 69, and 60% respectively. The peak time of the protective effect of PACAP was at reoxygenation 12-hour timepoint and then decreased. There existed no significant difference in MTT OD values between PACAP + OGD3h/R36h BV2 cells and control ($P>0.05$) (Fig. 1B and C). Then the effects of PACAP on BV2 microglial cells exposed to OGD/R were determined by TBE assay. Consistently with MTT assay, PACAP significantly increased cell viability in BV2 cells under OGD3h/R12h ($P<0.05$) (Fig. 1D).

PACAP exerted its neuroprotection to BV2 microglia in a dose-independent manner

Subsequently, to determine the neuoprotective effect of PACAP was whether dose-dependent or dose-independent, MTT assay was performed in BV2 cells after exposure to OGD for 3 hours followed by reoxygenation for 12 hours. We found that PACAP could significantly increase MTT OD value of OGD/R BV2 microglia by 32, 17, and 21% under different concentration of PACAP 10, 100, and 1000 nM respectively. There were no significant differences in neuroprotection of PACAP among the three different concentrations ($P>0.05$) (Fig. 2A). In general, PACAP may play a neuroprotective role in OGD/R BV2 microglia in a dose-independent manner. Then we investigated whether picomolar concentrations of PACAP could protect BV2 cells from cell death induced by OGD/R using MTT assay. We found that MTT OD value decreased significantly in OGD 3 hours followed by reoxygenation 12 hours ($P<0.05$). Cell viability significantly increased in BV2 cells under OGD/R when 100 pM PACAP was added ($P<0.05$), but no dramatic change occurred when 10 pM PACAP was added (Fig. 2B).

PACAP inhibited TLR4, MyD88 expression in BV2 microglial cells subjected to OGD followed by reoxygenation

To investigate the expression of TLR4 and MyD88, BV2 microglial cells were exposed to OGD for 15 minutes followed by reoxygenation for 12 hours. TLR4 and MyD88 protein levels were determined by Western blot analysis. As was shown in Fig. 3A, TLR4 expression was increased when BV2 microglial cells were exposed to OGD/R. PACAP treatment significantly inhibited upregulation of TLR4 expression induced by OGD/R ($P<0.05$, Fig. 3B). It was interesting that PACAP added into the control cells likewise induced downregulation of TLR4 protein expression (second panel).

OGD/R increased the expression of MyD88 protein in BV2, which was inhibited by PACAP pretreatment. MyD88 protein expression did not alter significantly when PACAP was added into the control BV2 cells ($P>0.05$) (Fig. 3).

PACAP inhibited IkappaB-alpha phosphorylation/degradation and NF-kappaB activation in BV2 microglial cells exposed to OGD followed by reoxygenation

To further dissect the downstream pathway of TLR4-mediated signal transduction, we next sought to determine the level of IkappaB-alpha phosphorylation and degradation in BV2 cells subjected to OGD/R and PACAP administration using western blot analysis. As shown in Fig. 4A, the expression of p-IkappaB-alpha increased and IkappaB-alpha decreased after exposure to OGD/R. PACAP significantly

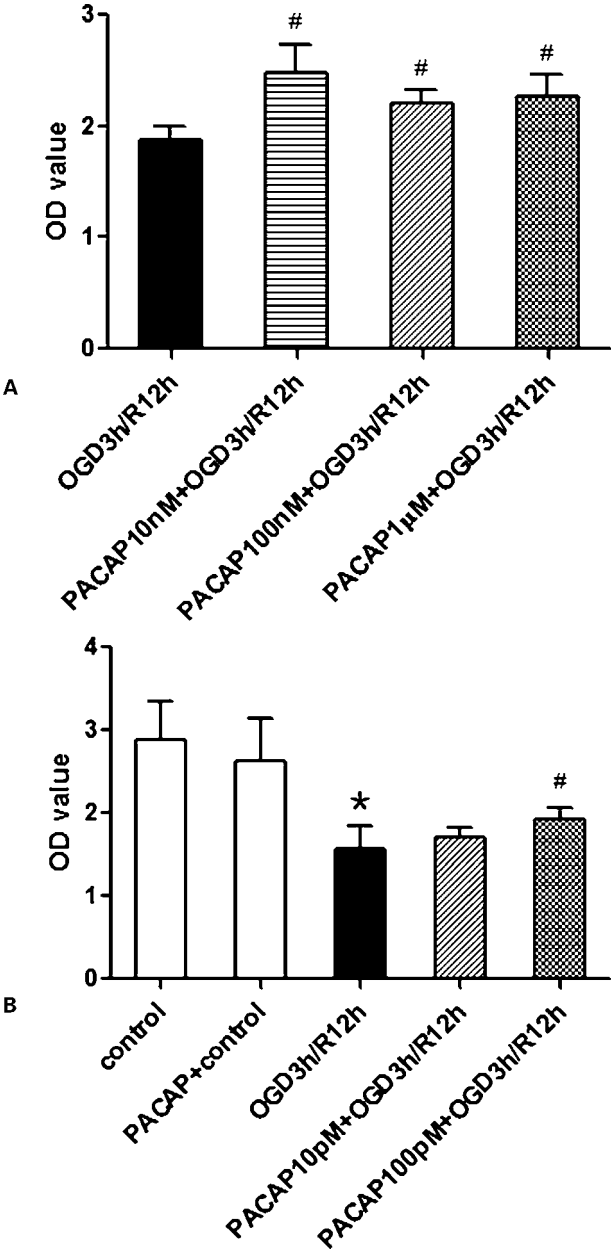

Figure 2 Effects of different dosage of PACAP on cell viability of BV2 microglia exposed to OGD/R. BV2 microglial cells were exposed to OGD for 3 hours followed by reoxygenation for 12 hours. (A) Cells were treated with the indicated concentration of PACAP 10, 100, and 1000 nM for immediately before OGD/R. (B) Cells were treated with picomolar concentrations of PACAP (10 and 100 pM) immediately before OGD/R. Cell viability was assessed using MTT assay, and the results are expressed as MTT OD value. Each value indicates the means ± SD and is representative of results obtained from four independent experiments. *$P<0.05$, as compared with control group. #$P<0.05$, as compared with OGD/R group.

inhibited phosphorylation of IkappaB-alpha in BV2 cells under OGD/R ($P<0.05$). No dramatic change in IkappaB-alpha phosphorylation/degradation was found between control cells and control treated by PACAP (second panel) ($P>0.05$) (Fig. 4B).

Next, we detected NF-kappaB p65 protein levels in nucleus. NF-kappaB was activated in the OGD/R

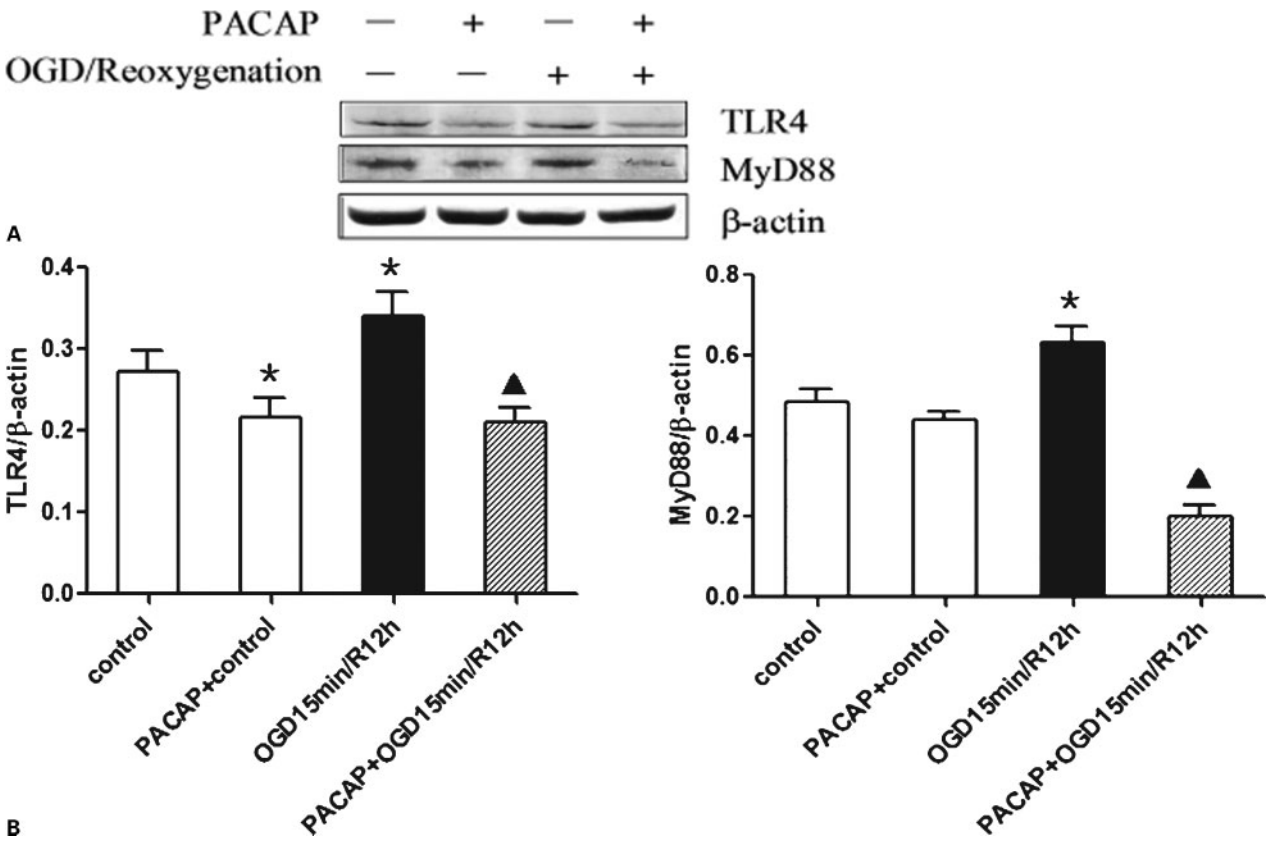

Figure 3 Induction of TLR4, MyD88 expression in the OGD/R BV2 microglia but inhibition due to PACAP. BV2 cells suffered from OGD/R by being exposed to an anerobic gas mixture (94% N_2, 5%CO_2, and 1% O_2) in glucose-free medium for 15 minutes and maintained at normoxia for 12 hours. PACAP (100 nM) was added into the medium immediately before OGD/R. Cell lysates were prepared and Western blot analyses were performed. (A) Cell lysates were immunoblotted with TLR4 and MyD88 antibodies (upper panel) and subsequently reprobed with beta-actin (lower level). These results are representative for two independent experiments. (B) The differences of the protein expression between the groups were analyzed with Image J. *$P<0.05$, as compared with control group. ▲$P<0.05$, as compared with OGD15min/R12h group.

BV2 cells, which was reversed by PACAP pretreatment ($P<0.05$). Surprisingly, when PACAP was added into the control BV2 cells, NF-kappaB protein levels were elevated (Fig. 4). Thus, inhibition of TLR4 signalling with PACAP contributes to neuroprotection in BV2 cells following OGD/R.

PACAP attenuated OGD/R induced apoptosis in BV2 microglial cells

MTT assay showed that PACAP played a modest cytoprotection after exposure to OGD3h/R12h; therefore, we detected apoptosis at this timepoint. Flow cytometer indicated that the percentage of apoptotic cells was increased after exposure to OGD/R, which was downregulated when PACAP was added ($P<0.05$). OGD/R induced apoptosis by 85% compared with the control, but PACAP decreased apoptosis by 28% compared with the OGD/R group and decreased apoptosis in PACAP-treated control cells (Fig. 5).

To investigate the mitochondrial apoptotic events involved in PACAP-reduced apoptosis, we analyzed the changes in Bcl-2 family members, which included pro-apoptotic protein (Bax) and anti-apoptotic protein (Bcl-2), two important regulators triggering mitochondrial depolarization in the process of apoptosis. Our results showed that OGD/R increased Bax/Bcl-2 ratio ($P<0.05$). Pretreatment with 100 nM PACAP downregulated the Bax level and upregulated the Bcl-2 protein level, resulting in a decrease in the Bax/Bcl-2 ratio ($P<0.05$) (Fig. 6). Thus, Bax and Bcl-2 played important roles in OGD/R induced apoptosis and PACAP induced neuroprotection.

PACAP reduced OGD/R induced TNF-alpha release in the BV2 supernatants

Since PACAP alleviated microglial hypoxic injury correlated with decreased protein levels of components of the TLR4/MyD88/NF-kappaB pathway, we hypothesized that a reduction in NF-kappaB mediated transcription and expression also occurred. To examine the effects of PACAP on cytokine secretion, TNF-alpha release, a proinflammatory cytokine was confirmed by ELISA. Our results showed that exposure to OGD3h/R12h enhanced TNF-alpha level by 51% in BV2 microglia. Pretreatment with PACAP suppressed TNF-alpha level by 37% compared with the OGD/R BV2 cells ($P<0.05$). As expected, PACAP significantly decreased the secretion of TNF-alpha proinflammatory cytokine. The

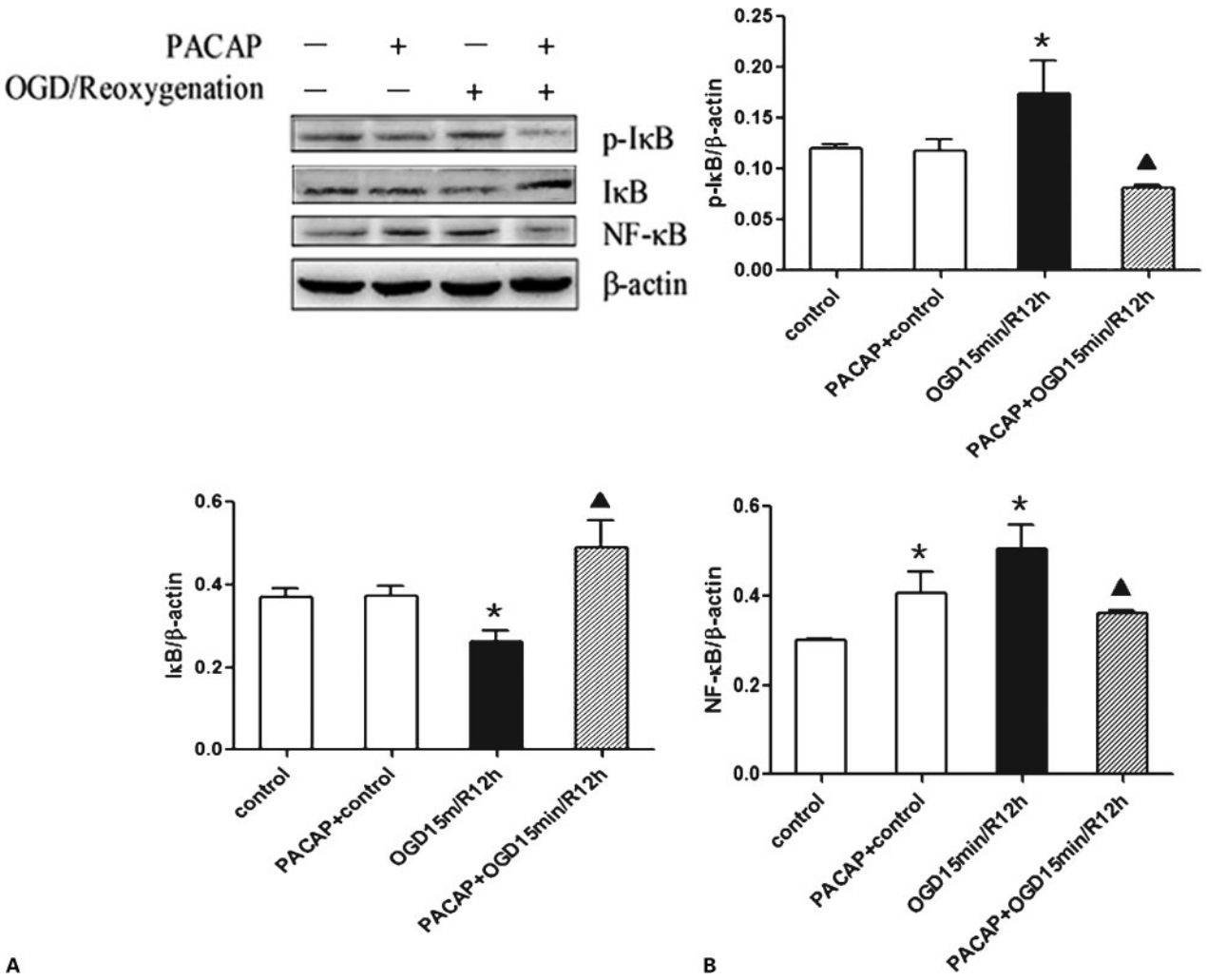

Figure 4 Induction of IkappaB phosphorylation and NF-kappaB activation by OGD/R in BV2 microglia but inhibition due to PACAP. BV2 cells were pretreated with 100 nM PACAP before OGD15min/R12h. (A) Total protein was extracted, and 100 μg of protein was subjected to SDS–PAGE followed by IkappaB-alpha, phospho-IkappaB-alpha, NF-kappaB, and beta-actin immunoblotting using the alkaline phosphatase technique. These results are representative for two independent experiments. (B) The differences of the protein expression between the groups were analyzed with Image J. *$P<0.05$ versus control group. ▲$P<0.05$ versus OGD/R group.

PACAP-treated control BV2 cells were not significantly different from the untreated control likewise ($P>0.05$) (Fig. 7).

Discussion

The present in vitro study demonstrated that administration of PACAP immediately before OGD/R, protected microglial cells from OGD/R injury. Pretreatment with PACAP inhibited activation of the TLR4/MyD88/NF-kappaB signaling pathway and decreased inflammatory cytokine levels as well as apoptosis in microglia, thereby attenuating microglial hypoxic injury. Our data suggested an important role of TLR4-mediated MyD88-dependent NF-kappaB signaling in response to the neuroprotective effects of PACAP to microglia against OGD/R injury.

Our first aim was to confirm whether PACAP could protect BV2 microglia from OGD/R injury. PACAP, originally isolated from the sheep hypohalamic extracts and described as a factor potently stimulating adenylyl cyclase activity in the rat pituitary cell culture. In the current study, we found that PACAP increased cell viability of BV2 microglia exposed to OGD followed by reoxygenation by MTT assay, morphology observation with light microscope and TBE assay. Firstly, we investigated the effects of different time duration of OGD on BV2 microglial cells. No dramatic change was found at OGD 3-hour time point, because no significant hypoxic injury occurred after exposure to OGD3h/R6h. OGD for 3 hours and reoxygenation for 24 hours triggered death in nearly 70% of cells.[26] The cell death rate of astrocytes significantly increased after 6 hours of OGD exposure followed by 24 hours of reoxygenation.[27] As most natural peptides, PACAP undergoes rapid enzymatic degradation after its introduction in the systemic circulation.[28] The half-life of PACAP38 in mice, estimated by tandemmass spectrometry, is less than 2 minutes.[29] A mean plasma half-life of 3.5 ± 1.3 minutes has been reported in a

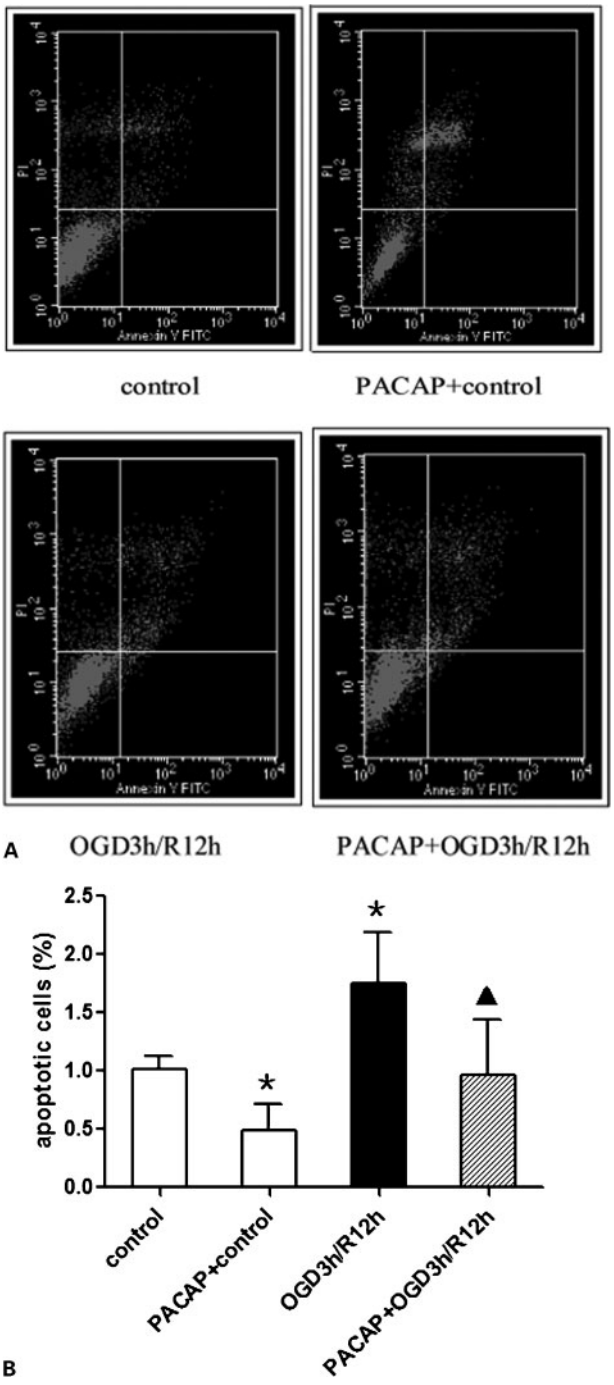

Figure 5 Effect of PACAP on OGD/R induced apoptosis in BV2 microglia. (A) After exposure to OGD for 3 hours and reoxygenation for 12 hours, BV2 cells were stained with annexin V and PI and analyzed by FACS. The apoptotic cells (the annexin V-positive and PI-negative cells) were indicated as the percentage of gated cells. (B) Relative percentage of the annexin V-positive and PI-negative cells. Data are the mean ± SD (n= 3). *P<0.05, as compared with control group. ▲P<0.05, as compared with OGD/R group.

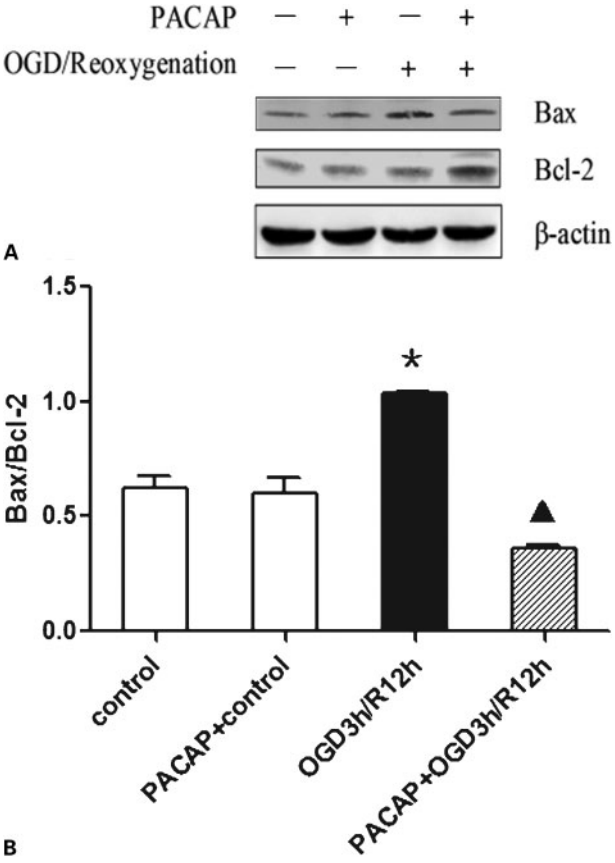

Figure 6 Augmentation of Bax/Bcl-2 in the OGD/R BV2 microglia and reduction of the ratio due to PACAP. BV2 cells were incubated with 100 nM PACAP followed by OGD for 3 hours and reoxygenation for 12 hours. (A) The cell lysates were immunoblotted with anti-Bax, anti-Bcl-2 or beta-actin antibodies. These results are representative for two independent experiments. (B) The differences of the protein expression between the groups were analyzed with Image J. *P<0.05 versus control group. ▲P<0.05 versus OGD/R group.

study involving 12 healthy young volunteers.[30] However, in our present *in vitro* study, PACAP could exert its neuroprotective effects until 36 hours. Thus secondly, the effects of OGD 3 hours followed by different time duration of reoxygenation on BV2 cells were detected. We found that pretreatment with PACAP in BV2 microglia subjected to OGD3h/R36h, there existed no significant difference in MTT OD value compared with control, which suggested that cell viability of BV2 cells nearly recovered to normal level due to enough reoxygenation (36 hours) and the protective effect of PACAP. Pretreatment with PACAP for 3 hours of OGD and 12 hours of reoxygenation, BV2 microglial cell edema and cell death were significantly ameliorated and MTT activity was dramaticly increased. Therefore, we chose this OGD and reoxygenation exposure time for the subsequent flow cytometry and ELISA studies. Thirdly, we demonstrated that PACAP exerted its neuroprotection to BV2 microglia in a dose-independent manner at the concerntration of 10, 100, and 1000 nM. And PACAP with concerntration of 100 pM could also protect BV2 cells from cell death induced by OGD/R, while 10 pM PACAP had no protective effect on BV2 microglia from cell death induced by OGD/R. Moreover, MTT activity and light microscope observation were not altered when PACAP was added into control BV2 cells, which suggested that PACAP had no influences on control BV2 cells.

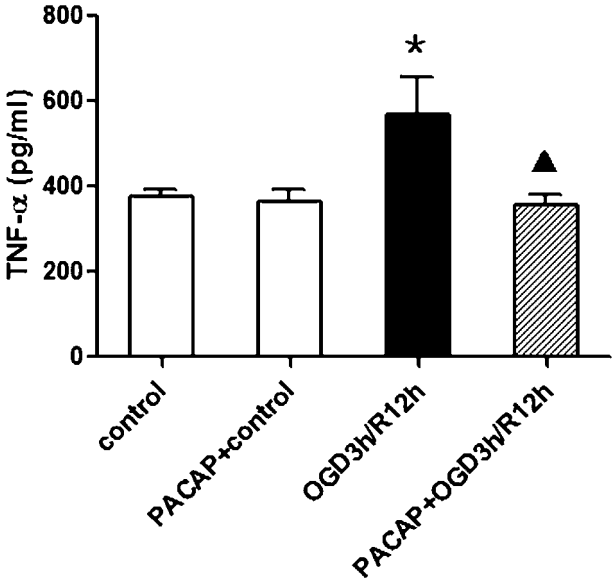

Figure 7 Induction of TNF-alpha release in the BV2 supernatants but inhibition of TNF-alpha release due to PACAP. Compartmentalized secretion of TNF-alpha was measured in the supernatants from BV2 cells subjected to OGD/R and PACAP-treated cells (100 nM). Data are shown as means ± SD (n=4). *P< 0.05, as compared with control group. ▲P<0.05, as compared with OGD/R group.

Subsequently, we attempted to explore the mechanism by which PACAP exerted neuroprotection to BV2 microglial cells against OGD/R. OGD/R resulted in microglia activation, which may exert deleterious or beneficial effects. A delicate equilibrium of microglial-derived factors might determine the neurotrophic or neurotoxic effect of activated microglia. Microglia-derived inflammatory factors such as TNF-alpha and IL-1beta trigger harmful downstream signaling pathways and accelerate neuronal death.[31] In the present study, microglia activation could be harmful resulting in cell death, which was consistent with the studies.[32,33] Activated microglia certainly appear to be toxic and inexorably exacerbate the death of dopaminergic neurons.[33] PACAP elicits a broad spectrum of biological effects on innate and acquired immunity.[34,35] PACAP38 suppressed TLR4 and proinf lammatory cytokine expression in kidneys subjected to ischemia/reperfusion (I/R) injury.[36] There might be many different ways in which mediators might activate microglia including fractalkine, interferon-gamma, monocyte chemoattractant protein-1, P2X4, and TLR4.[37] TLRs play a considerable role in the induction of innate immune and inflammatory responses. TLR activation contributed to cerebral I/R injuries.[38] Lehnardt *et al.*[39] reported that TLR4 activation led to extensive neuronal death *in vitro* that depended on the presence of microglia. Tang *et al.*[40] reported that TLR2 and -4 expression was increased in cerebral cortical neurons in response to I/R injury. Hypoxia upregulated TLR4 expression at the mRNA and protein levels in

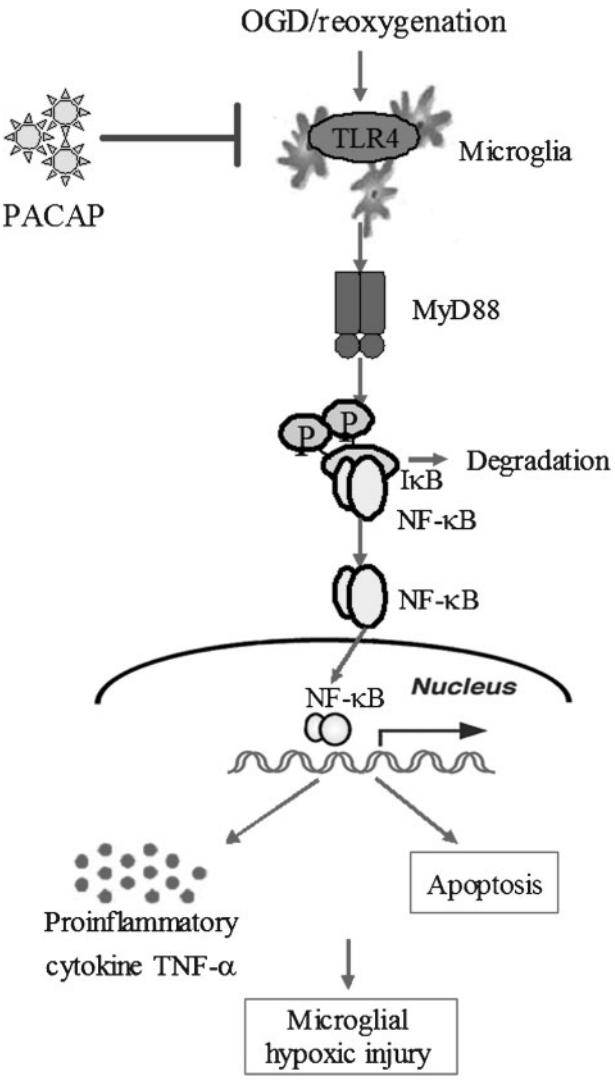

Figure 8 Schematic diagram depicting proposed mechanisms for TLR4-mediated neuroprotection of PACAP to BV2 microglia against OGD/R. PACAP decreased TLR4 expression and downstream MyD88 expression and NF-kappaB activation. In addition, downregulation of apoptosis and proinflammatory cytokine secretion were involved in neuroprotection of PACAP to microglia against the hypoxic injury. → stimulatory modification; ⊣ inhibition.

microglia and regulated MyD88-dependent and -independent pathways of TLR4 signaling.[41] In addition, activated microglia can proliferate, potentiate phagocytosis, actively migrate to the site of injury, and release a variety of factors including cytokines, chemokines and nitric oxide, thereby causing an inflammatory response. Therefore, it was possible that activation of TLR4 in microglia could mediate inflammatory response, blockade of which could reduce microglia ischemic/hypoxic injury. In the current study, OGD/R resulted in TLR4 activation, which was inhibited by PACAP treatment.

Presumably, the TLR4 activated downstream signaling pathway, including MyD88 and NF-kappaB activation, can promote microglia activation and the production of proinflammatory cytokines as part of the response to OGD/R. Based upon the ability of

PACAP to attenuate the increase in TLR4 protein levels, we expect that a component of its neuroprotective benefits is attenuation of TLR4-dependent signaling in microglia. Li *et al.*[42] reported that TLR4-mediated cytokine responded to hypoxia are primarily dependent on MyD88 expression and MyD88-dependent mechanisms in the coordination of the innate immune plays pivotal role in ischemic/hypoxic acute renal tubular injury. Wang *et al.*[15] reported that MyD88 played an essential role in LPS-sensitized HI neonatal brain injury, which involved both inflammatory and caspase-dependent pathways. We observed in the present study administration of PACAP immediately before OGD/R resulted in marked inhibition of OGD/R induced TLR4 and MyD88 expression in BV2 microglia. Surprisingly, PACAP pretreatment could inhibit TLR4 and MyD88 expression to the level lower than control.

It has been well established that NF-kappaB is a key regulator in general inflammatory, as well as immune response.[43,44] In the resting state, IkappaB-alpha continues to be synthesized in the body, but most molecules are quickly degraded and leave only a small part of free protein and the dimeric form (typically RelA/p65 and p50) of NF-kappaB is trapped in the cytoplasm by IkappaB proteins.[45,46] NF-kappaB activation was involved in cerebral ischemia *in vivo* and *in vitro*,[47] which was consistent with our results showing that PACAP inhibited NF-kappaB activation in BV2 microglia exposed to OGD followed by reoxygenation. NF-kappaB was involved in apoptosis and NF-kappaB activation may be pro-apoptotic.[48,49] Given the fact that oxidative stress can induce apoptosis and the important role that apoptosis has in the pathogenesis of hypoxia/reperfusion injury,[50,51] we examined the effects of PACAP on OGD/R induced apoptosis in BV2 microglia. Our results suggested that PACAP inhibited the intrinsic pathway of apoptosis induced by OGD/R. Microglia is the primary producers of TNF-alpha in the brain and may play a role in many pathological conditions in the brain.[52] TNF-alpha overexpression has been implicated in the pathogenesis of several human CNS disorders.[53,54] Our data indicated that treatment with PACAP before OGD/R resulted in significantly attenuated production of TNF-alpha in BV2 microglia. Taken together, these results documented involvement of NF-kappaB, apoptosis, and proinflammatory cytokine secretion in the inhibitory effects of PACAP on OGD/R.

In summary, PACAP could protect BV2 microglia cells against hypoxic injury in dose-independent manner. Then correlative evidence supported a mechanism that PACAP inhibited upregulation of TLR4 expression and its downstream MyD88 expression and NF-kappaB activation. In addition, inhibition of apoptosis and proinflammatory cytokine secretion were involved in neuroprotection of PACAP to microglia. Our results suggested that TLR4-mediated MyD88-dependent signaling contributed to BV2 OGD/R injury and that inhibition of the signaling pathway could be a new therapeutic strategy for protection of brain against ischemic injury, suggesting that PACAP be considered a potential candidate for clinical trials in brain ischemic diseases.

Acknowledgements

This project was supported by grants from the National Natural Science of China (81171041), the Natural Science foundation of Jiangsu Province (BK2011197), the Social Development Science and Technology Project of Xuzhou city (xzzd1051), Educational Department Science Research Foundation of Jiangsu Province (09KJD310009), the Project of Xuzhou Medical College (2010KJ07) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions of Jiangsu Province.

References

1 Deng YY, Lu J, Ling EA, Kaur C. Role of microglia in the process of inflammation in the hypoxic developing brain. Front Biosci (Schol Ed). 2011;3:884–900.
2 Kim SU, de Vellis J. Microglia in health and disease. J Neurosci Res. 2005;81(3):302–13.
3 Arimura A. Perspectives on pituitary adenylate cyclase-activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol. 1998;48:301–31.
4 Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H. Pituitary adenylate cyclase activating polypeptide and its receptors: from structure to functions. Pharmacol Rev. 2000; 52:269–324.
5 Lenti L, Zimmermann A, Kisa D, Oláh O, Tóth GK, Hegyi O, *et al.* PACAP and VIP differentially preserve neurovascular reactivity after global cerebral ischemia in newborn pigs. Brain Res. 2009;1283:50–7.
6 Stetler RA, Gao Y, Zukin RS, Vosler PS, Zhang L, Zhang F, *et al.* Apurinic/apyrimidinic endonuclease APE1 is required for PACAP-induced neuroprotection against global cerebral ischemia. Proc Natl Acad Sci USA. 2010;107(7):3204–9.
7 Dejda A, Seaborn T, Bourgault S, Touzani O, Fournier A, Vaudry H, *et al.* PACAP and a novel stable analog protect rat brain from ischemia: insight into the mechanisms of action. Peptides. 2011;32:1207–16.
8 Reglodi D, Tamás A, Somogyvári-Vigh A, Szántó Z, Kertes E, Lénárd L, *et al.* Effects of pretreatment with PACAP on the infarct size and functional outcome in rat permanent focal cerebral ischemia. Peptides. 2002;23:2227–34.
9 Reglodi D, Kiss P, Lubics A, Tamas A. Review on the protective effects of PACAP in models of neurodegenerative diseases *in vitro* and *in vivo*. Curr Pharm Des. 2011;17(10):962–72.
10 Vaudry D, Gonzalez BJ, Basille M, Pamantung TF, Fontaine M, Fournier A, *et al.* The neuroprotective effect of pituitary adenylate cyclase activating polypeptide on cerebellar granule cells is mediated through inhibition of the CED3-related cysteine protease caspase-3/CPP32. Proc Natl Acad Sci USA. 2000;97:13390–5.
11 Vaudry D, Rousselle C, Basille M, Falluel-Morel A, Pamantung TF, Fontaine M, *et al.* Pituitary adenylate cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death. Proc Natl Acad Sci USA. 2002;99:6398–403.
12 Dejda A, Nowak JZ. Neuroprotective potential of three neuropeptides PACAP, VIP and PHI. Pharmocol Reports. 2005;57:307–20.

13 Kyoungho Suk, Park JH, Lee WH. Neuropeptide PACAP inhibits hypoxic activation of brain microglia: a protective mechanism against microglial neurotoxicity in ischemia. Brain Res. 2004;1026:151–6.
14 Serebrovskaya TV, Nikolsky IS, Nikolska VV, Mallet RT, Ishchuk VA. Intermittent hypoxia mobilizes hematopoietic progenitors and augments cellular and humoral elements of innate immunity in adult men. High Alt Med Biol. 2011; 12(3):243–52.
15 Wang X, Stridh L, Li W, Dean J, Elmgren A, Gan L, *et al.* Lipopolysaccharide sensitizes neonatal hypoxic-ischemic brain injury in a MyD88-dependent manner. J Immunol. 2009; 183(11);7471–7.
16 Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. Lancet. 2011;378(9795):1015–26.
17 Beutler B. Inferences, questions and possibilities in Toll-like receptor signaling. Nature. 2004;430:257–63.
18 Rakoff-Nahoum S, Medzhitov R. Role of Toll-like receptors in tissue repair and tumorigenesis. Biochemistry (Mosc). 2008; 73:555–61.
19 Cao Z, Hu Y, Wu W, Ha T, Kelley J, Deng C, *et al.* The TIR/BB-loop mimetic AS-1 protects the myocardium from ischaemia/reperfusion injury. Cardiovasc Res. 2009;84(3):442–51.
20 Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol. 2005;17(1):1–14.
21 Chearwae W, Bright JJ. 15-deoxy-delta(12,14)-prostaglandin J(2) and curcumin modulate the expression of Toll-like receptors 4 and 9 in autoimmune T lymphocyte. J Clin Immunol. 2008;28:558–70.
22 O'Neill LA, Bryant CE, Doyle SL. Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol Rev. 2009;61:177–97.
23 Velayudham A, Hritz I, Dolganiue A, Mandrekar P, Kurt-Jones E, Szabo G. Critial role of Toll- like receptors and the common TLR adaptor, MyD88, in induction of granulomas and liver injury. J Hepatol. 2006;45(6):813–24.
24 Yang YX, Zhou HD, Yang YB, Li W, Zhou M, Zeng Z, *et al.* Lipopolysaeeharide (LPS) regulates TLR4 signal transduction in nasopharynx epithelial cell line 5-8F via NF-kappaB and MAPKS signaling pathways. Mol Immunol. 2007;44(5):984–92.
25 Zwagerman N, Plumlee C, Guthikonda M, Ding Y. Toll-like receptor-4 and cytokine cascade in stroke after exercise. Neurol Res. 2010;32(2):123–6.
26 Wang ZF, Zhou J, Tang XC. Huperzine B protects rat pheochromocytoma cells against oxygen-glucose deprivation-induced injury. Acta Pharmacol Sin. 2002;23(12):1193–8.
27 Zhu SM, Xiong XX, Zheng YY, Pan CF. Propofol inhibits aquaporin 4 expression through a protein kinase C-dependent pathway in an astrocyte model of cerebral ischemia/reoxygenation. Anesth Analg. 2009;109(5):1493–9.
28 Bourgault S, Vaudry D, Dejda A, Doan ND, Vaudry H, Fournier A. Pituitary adenylate cyclase-activating polypeptide: focus on structure-activity relationships of a neuroprotective peptide. Curr Med Chem. 2009;16(33):4462–80.
29 Zhu L, Tamvakopoulos C, Xie D, Dragovic J, Shen X, Fenyk-Melody JE, *et al.* The role of dipeptidyl peptidase IV in the cleavage of glucagon family peptides: *in vivo* metabolism of pituitary adenylate cyclase activating polypeptide-(1–38). J Biol Chem. 2003;278(25):22418–23.
30 Birk S, Sitarz JT, Petersen KA, Oturai PS, Kruuse C, Fahrenkrug J, *et al.* The effect of intravenous PACAP38 on cerebral hemodynamics in healthy volunteers. Regul Pept. 2007;140(3):185–91.
31 Yun HJ, Yoon JH, Lee JK, Noh KT, Yoon KW, Oh HJ, *et al.* Daxx mediates activation-induced cell death in microglia by triggering MST1 signalling. EMBO J. 2011;30(12):2465–76.
32 Taylor DL, Pirianov G, Holland S, McGinnity CJ, Norman AL, Reali C, *et al.* Attenuation of proliferation in oligodendrocyte precursor cells by activated microglia. J Neurosci Res. 2010;88(8):1632–44.
33 Cao JJ, Li KS, Shen YQ. Activated immune cells in Parkinson's disease. J Neuroimmune Pharmacol. 2011;6:323–9.
34 Delgado M, Abad C, Martinez C, Juarranz MG, Leceta J, Ganea D, *et al.* PACAP in immunity and inflammation. Ann NY Acad Sci. 2003;992:141–57.
35 Ganea D, Delgado M. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. Crit Rev Oral Biol Med. 2002;13(3):229–37.
36 Li M, Khan AM, Maderdrut JL, Simon EE, Batuman V. The effect of PACAP38 on MyD88-mediated signal transduction in ischemia-/hypoxia-induced acute kidney injury. Am J Nephrol. 2010;32(6):522–32.
37 Smith HS. Activated microglia in nociception. Pain Physician. 2010;13(3):295–304.
38 Ben Mkaddem S, Pedruzzi E, Werts C, Coant N, Bens M, Cluzeaud F, *et al.* Heat shock protein gp96 and NAD(P)H oxidase 4 play key roles in Toll-like receptor 4-activated apoptosis during renal ischemia/reperfusion injury. Cell Death Differ. 2010;17:1474–85.
39 Lehnardt SL, Massillon P, Follett FE, Jensen FE, Ratan R, Rosenberg PA, *et al.* Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc Natl Acad Sci USA. 2003;100:8514.–9.
40 Tang SC, Arumugam TV, Xu X, Cheng A, Mughal MR, Jo DG, *et al.* Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. Proc Natl Acad Sci USA. 2007;104(34):13798–803.
41 Ock J, Jeong J, Choi WS, Lee WH, Kim SH, Kim IK, *et al.* Regulation of Toll-like receptor 4 expression and its signaling by hypoxia in cultured microglia. J Neurosci Res. 2007; 85(9):1989–95.
42 Li M, Khan AM, Maderdrut JL, Simon EE, Batuman V. The effect of PACAP38 on MyD88-Mediated signal transduction in ischemia-/hypoxia-induced acute kidney injury. Am J Nephrol. 2010;32(6):522–32.
43 Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008;132(3):344–62.
44 Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Ann Rev Immunol. 2009;27:693–733.
45 Solt LA, May MJ. The IkappaB kinase complex: master regulator of NF-kappaB signaling. Immunol Res. 2009;42(1–3):3–18.
46 Jeon J, Park KA, Lee H, Shin S, Zhang T, Won M, *et al.* Water extract of Cynanchi atrati Radix regulates inflammation and apoptotic cell death through suppression of IKK-mediated NF-kappaB signaling. J Ethnopharmacol. 2011;137:626–34.
47 Jiang W, Fu F, Tian J, Zhu H, Hou J. Curculigoside A attenuates experimental cerebral ischemia injury *in vitro* and *vivo*. Neuroscience. 2011;192:572–9.
48 Wang F, Li H, Shi H, Sun B. Pro-apoptotic role of nuclear factor-kappaB in adriamycin-induced acute myocardial injury in rats. Mol Med Report. 2012;5(2):400–4.
49 Wang H, Cho CH. Effect of NF-kappaB signaling on apoptosis in chronic inflammation-associated carcinogenesis. Curr Cancer Drug Targets. 2010;10(6):593–9.
50 Yang YF, Chen Z, Hu SL, Hu J, Li B, Li JT, *et al.* Interleukin-1 receptor associated kinases-1/4 inhibition protects against acute hypoxia/ischemia-induced neuronal injury *in vivo* and *in vitro*. Neuroscience. 2011;196:25-34.
51 Chu W, Li X, Li C, Wan L, Shi H, Song X, *et al.* TGFBR3, a potential negative regulator of TGF-beta signaling, protects cardiac fibroblasts from hypoxia-induced apoptosis. J Cell Physiol. 2011;226(10):2586–94.
52 Sawada M, Kondo N, Suzumura A, Marunouchi T. Production of tumornecrosis factor-alpha by microglia and astrocytes in culture. Brain Res. 1989;491(2):394–7.
53 Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI, O'Callaghan JP. Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. FASEB J. 2002;16(11):1474–6.
54 Cheong MH, Lee SR, Yoo HS, Jeong JW, Kim GY, Kim WJ, *et al.* Anti-inflammatory effects of *Polygala tenuifolia* root through inhibition of NF-kappaB activation in lipopolysaccharide-induced BV2 microglial cells. J Ethnopharmacol. 2011;137(3):1402–8.